



# Annals of Biochemistry and Experimental Medicine

Volume IX, 1949

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## ERRATA

Vol. VIII, No. 3-4, 1948, Page 96, Line 11.

For "pepsin" read "trypsin".



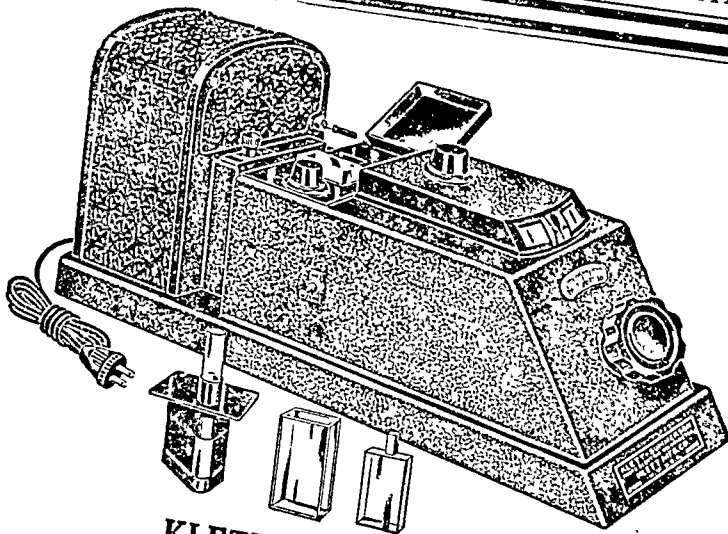
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**A SHORTENED PROCEDURE FOR ESTIMATING VITAMIN B<sub>1</sub>  
IN FOODSTUFFS**

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Harris and Wang (1) described a method for estimating vitamin B<sub>1</sub> in foodstuffs and biological materials by the thiochrome test. The method is admirably suited to the routine determination of vitamin B<sub>1</sub> in foodstuffs and recently the vitamin B<sub>1</sub> Sub-Committee (2) of the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute, London recommended the method for adoption in routine determination of vitamin B<sub>1</sub> in national flour. The various steps involved in the method are:—

- (a) extraction of vitamin on boiling water-bath for 10 minutes.
- (b) digestion with a mixture of papain and taka-diastase at 40-45°C. for 16-18 hours.
- (c) removal of fluorescent materials by washing with isobutanol.
- (d) development of thiochrome.
- (e) visual comparison of fluorescence.

It is clear from the above that the time taken for carrying out each estimation is more than 18-20 hours. For routine determinations involving large number of samples, it is advantageous to shorten the time taken for the determination of vitamin to a bare minimum and the work to be described was undertaken with this object in view. The modification introduced in the method involves the use of high temperature (60-63°C.) for digestion, thereby shortening the period of digestion to about one hour instead of 18-20 hours according to Harris and Wang's method. The results obtained by the modified method agreed well with those determined by Harris and Wang's method.

## EXPERIMENTAL.

Weighed amount (0.5-2.0 g. depending upon the vitamin B<sub>1</sub> content) of the sample was taken in a conical flask and 0.1 g. of papain (prepared in the laboratory from fresh latex) was added followed by 15 cc. of 0.02 N. HCl. The pH was adjusted to 4.0-4.5 and kept for digestion at 60-63°C. for one hour and then transferred to a 25 cc. volumetric flask and made up to the mark. The rest of the procedure was exactly similar to that described in the original method of Harris and Wang (1).

Vitamin B<sub>1</sub> determinations in each sample were carried out by Harris and Wang's method and the present modified method. The results are summarised in Table I.

TABLE I

*Comparison of results obtained by Harris and Wang's method and the present modified method.*

MATERIAL	Vitamin B <sub>1</sub> content $\mu$ /g.	
	Vitamin B <sub>1</sub> Sub-Committee method.	Modified method.
I. CEREALS.		
<i>Oryza sativa</i>		
1. Sela rice <sup>1</sup> ( <i>Basumati</i> )	2.5	2.4
2. Raw rice	2.8	2.9
<i>Wheat (Triticum vulgare)</i>		
3. Whole wheat ( <i>Triticum vulgare</i> )	3.2	3.0
4. Wheat flour	2.9	2.9
5. Ragi ( <i>Eleusine coracana</i> )	2.5	2.3
6. Cholam ( <i>Sorghum vulgare</i> )	3.6	3.7
II. PULSES.		
7. Black gram ( <i>Phaseolus mungo</i> )	4.5	4.7
8. Horse gram ( <i>Dolichos biflorus</i> )	4.2	4.1
9. Green gram ( <i>Phaseolus radiatus</i> )	3.5	3.3
10. Cowpea ( <i>Vigna catiang</i> )	3.8	3.7
III. OIL SEEDS.		
<i>Groundnut (Arachis hypogea)</i>		
11. A.H.32 (defatted flour fat content 46.17%) Coimbatore	20.0	20.5
12. A H.25 (Coimbatore) flour defatted, fat content 49.5%	16.5	16.5
13. A.II.698 (Coimbatore, flour defatted, fat content 47.0%)	19.1	19.2
14. Sesame ( <i>sesamum indicum</i> ) flour defatted, fat content 48.1%)	9.5	9.5

1. Prepared by a special form of parboiling process known as *sela*-process, widely adopted in Punjab and other parts of Northern India. Briefly stated the process consists in steeping the paddy in water for 24-48 hours and then gently roasted in hot sand in an iron pan. This form of rice is said to be superior in certain respects to ordinary parboiled rice.

## DISCUSSION.

The good agreement of the results obtained by the present modified method compared with the original Harris and Wang's method appears to establish its reliability. Furthermore, it can be seen from the results that no significant differences were noticed in the values obtained by digesting the material with papain alone or with a mixture of taka-diastase and papain as recommended by Harris and Wang. This observation clearly shows that vitamin B<sub>1</sub> exists in phosphorylated form mostly in nonphosphorylated form and even if it exists in phosphorylated form as cocarboxylase, it may be present in very minute quantities which may be easily hydrolysed during extraction to free form by the phosphatase, the presence of which appears to be almost universal in vegetable materials (Giri, 3).

In the case of groundnut about 50-60% of vitamin B<sub>1</sub> exists in bound form, which cannot be extracted unless it is digested with papain and in such cases the vitamin B<sub>1</sub> is probably bound to protein. Our preliminary studies on groundnut have further shown that the bound form of vitamin B<sub>1</sub> is rendered free during germination. Further investigations on the isolation, properties and physiological function of the bound form of vitamin B<sub>1</sub> are in progress.

## SUMMARY

1. A shortened procedure for the estimation of vitamin B<sub>1</sub> in food stuffs, which is a modification of the method of Harris and wang, is described.
2. The modification consists in shortening the period of digestion by carrying out the digestion with papain at 60-63°C. for one hour.
3. The values obtained for vitamin B<sub>1</sub> content of foodstuffs by the present modified method agree well with those determined by the original Harris and Wang's method.

## ACKNOWLEDGEMENT

Our thanks are due to Prof. V. Subrahmanyam for his keen interest in the present investigation.

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STUDIES IN COMPOUNDS OF THE DDT TYPE. PART I

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In a previous communication (1) the condensation of chloral with chloro-, bromo-, and iodo-benzene was described. The compounds thus obtained were analogous to DDT and their action on malarial larvae was also similar to that of the well-known insecticide. In the course of a systematic investigation in this line, chloral had been condensed with symmetrical trichloro-, tribromo- and triiodo-benzene. The activity of the resulting compounds was found to diminish with the increase in molecular weight, thus indicating that the nuclear halogen atoms did not contribute to an appreciable extent in conferring the required physiological property on this type of compounds. Next an attempt was made to study the properties of compounds having bromine in the side-chain in place of chlorine. The compounds obtained in this connection by the condensation of bromal with mono-halogen benzenes were found to be inactive against malarial larvae, an observation which was considered to be very interesting, in view of the observations of Mylius and Koechlin (2) namely that the action of the condensation product of bromal with chlorobenzene was somewhat slow in the case of flies (*Calliphora vomitoria*), while against moths (*Tincola biselliella*), the substance was not of much value. To complete this series of experiments, it was considered desirable to condense tri-iodo-acetaldehyde with halogen substitution products of benzene. But unfortunately this experiment could not be made on account of the extreme instability of triiodoacetaldehyde. Even mono-iodo acetaldehyde, which has been described in the literature, was found to decompose easily. Attempts are now being made to substitute the hydrogen atoms in the sidechain of the condensation product of acetaldehyde and chlorobenzene with iodine.

In studying the action of these compounds on malarial larvae some marked differences from DDT were noted. The condensation products of bromal with chloro- and bromo-benzenes were found to be soluble in kerosene, but the corresponding iodo-compound was insoluble in kerosene but soluble in a mixture of kerosene and xylene (1:1). The spreading of the solutions was also found to be different. A 5% kerosene solution of DDT was found to spread to a diameter of about 8 inches in water, but with dichloro-diphenyl-tribromoethane the diameter was found to be about 4 inches and the corresponding bromo- and iodo-compounds did not spread at all. When, however, a drop of castor oil was added, each of

these was found to spread to a diameter of 8 inches. These solutions, even with the addition of castor oil, were found to be without any action on malarial larvae in 24-48 hours; whereas in the case of DDT the larvae were usually killed in 4 to 6 hours. From these observations it might be safely concluded that the activity of these series of compounds depends upon the chlorine atom in the side-chain and the replacement of these by bromine destroys the activity. The nuclear halogen atoms do not appear to have any specific actions.

#### EXPERIMENTAL.

*Condensation of bromal and chlorobenzene.*—Bromal (21 g.), chlorobenzene (12 g.), sulphuric acid (50 cc., sp. gr 1.84) and fuming sulphuric acid (20%  $\text{SO}_3$ , 13 g.) were mixed in a three-necked round-bottom flask of 500 cc. capacity fitted with a mercury-sealed mechanical stirrer, a condenser with guard-tube and a thermometer. The initial temperature was  $25^\circ$ . The temperature quickly rose to  $40^\circ$  on stirring and was maintained at  $45\text{--}55^\circ$ . Granular crystals came down after 40 minutes and the stirring was continued for two hours more. The whole mass was then poured over crushed ice and left in a refrigerator. After 24 hours the clear liquid from the top was decanted off and the semi-solid mass was washed thoroughly with water. It was then stirred vigorously with hot water. This process was repeated twice. The crystals were then filtered, washed and pressed between filter papers to remove the adhering oil. The impure product was then crystallised from rectified spirit (charcoal) and finally from absolute alcohol in white needles, m.p.  $137^\circ$ . Yield 8 g.

(Found: Cl, 15.12; Br, 48.37,  $\text{C}_{11}\text{H}_5\text{Cl}_2\text{Br}_3$  requires Cl, 14.55; Br, 49.18%)

*Condensation of bromal and bromobenzene.*—Bromal (14 g.), bromobenzene (16 g.), sulphuric acid (50 cc., sp.gr. 1.84) and fuming sulphuric acid (20%  $\text{SO}_3$ , 18 g.) were treated in the manner stated above for 3 hours, the temperature being  $55\text{--}65^\circ$ . The solid was filtered off, washed with hot water filtered and pressed between filter papers. A portion was crystallised from absolute alcohol (charcoal). White needles, m.p.  $168^\circ$ .

(Found: Br, 68.23.  $\text{C}_{11}\text{H}_5\text{Br}_3$  requires Br, 69.32%).

*Condensation of bromal and iodobenzene.*—Bromal (12 g.), iodobenzene (13 g.), sulphuric acid (50 cc., sp. gr 1.84) and fuming sulphuric acid (20%  $\text{SO}_3$ , 20g.) were treated as described above for 6 hours at  $55\text{--}60^\circ$ . The reddish-brown sticky mass was treated with hot water and then dried between filter papers. The crude mass was treated with hot water and then dried between filter papers. The crude product was crystallised from cyclohexane, as colourless crystals, m.p.  $180^\circ$ . (Found: Br, 35.43; I, 38.01.  $\text{C}_{11}\text{H}_5\text{Br}_2\text{I}_2$  requires Br, 35.76; I, 37.85%).

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## STUDIES IN COMPOUNDS OF THE DDT TYPE. PART II

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The present communication deals with the condensation of sym-trichloro-, tribromo- and triiodo-benzene with chloral. The object was to prepare compounds of DDT type, having six nuclear halogen atoms.

The condensation of these halogen substituted benzenes with chloral presented some difficulty on account of their low solubility in concentrated sulphuric acid. In the case of the bromo-compound, the condensation was rendered more easy by dissolving it in pyridine and then proceeding in the usual manner. It was found that in the absence of fuming sulphuric acid the condensation did not take place.

### EXPERIMENTAL

#### *Preparation of sym. tribromobenzene:*

2:4:6 tribromoaniline, in fine powder (5 g.), was dissolved in alcohol with heating. Solid sodium nitrite (3 g.) was next added to the boiling solution. To this mixture was then added dilute sulphuric acid from a dropping funnel, with constant stirring, the rate of addition being just sufficient to keep the liquid boiling. A white solid separated during the initial stages which became brown towards the end of the operation. When the liquid was just acid in reaction, the addition of acid was stopped with the whole was allowed to stand overnight. The precipitate was filtered, washed with water thoroughly and crystallised from dilute alcohol (charcoal) in yellowish-white needles. M.P. 119°C. Yield-3.7 g.

#### *Preparation of 2:4:6:2':4':6'-hexachloro diphenyl trichloroethane:*

2:4:6-trichlorobenzene (8 g.) was dissolved in calculated sulphuric acid (about 100 cc.) in a three-necked flask provided with a thermometer, condenser and liquid-sealed mechanical stirrer. Fuming sulphuric acid (20% SO<sub>3</sub>, 6 cc.) was next added, followed by chloral (4 g.). The whole was vigorously stirred for 12 hours at a temperature between 40-45°C. At the end of this period, a solid separated and the liquid was poured over crushed ice. A brown pasty mass was then obtained which was thoroughly washed with water and dried on a porous plate. The crude product was crystallised several times from dilute alcohol



(charcoal) when a white needle-shaped solid was obtained. M.P.  $100^{\circ}\text{C}$ . Yield-0.8 g.

(Found Cl-65.23%:  $\text{C}_{11}\text{H}_5\text{Cl}_6$  requires Cl-65.10%).

*Properties.*—The substance is insoluble in water, hot and cold, and also dilute acids and alkalies. It is fairly soluble in hydrocarbon solvents and in dilute alcohol on heating. It is also soluble, with difficulty, in acetic acid and easily in chloroform, ether and carbon tetrachloride.

*Preparation of 2:4:6:2':4':4' hexabromo diphenyl trichloroethane:*

In this preparation, tribromobenzene (5 g.) was first of all dissolved in pyridine (about 20 cc.). The quantity of sulphuric acid added was about 150 cc. and fuming sulphuric acid (20%  $\text{SO}_3$ ) 20 cc. The temperature maintained during the condensation was about  $50^{\circ}\text{C}$  and the time allowed was 10 hours. The reaction mixture was then poured on crushed ice and allowed to stand overnight in a refrigerator. The acid liquid was decanted off, the lower pasty mass washed with water and finally crystallised from alcohol. M.P.  $121-22^{\circ}\text{C}$ . Yield-2.5 g.

(Found Cl-13.82%, Br-63.29%:  $\text{C}_{11}\text{H}_5\text{Cl}_3\text{Br}_6$  requires Cl-13.94%, Br-63.20%).

*Properties.*—Faint brown needles, insoluble in water and dilute acids and alkalies both hot and cold. Soluble in petrol, benzene, xylene, carbon tetrachloride, difficultly soluble in acetic acid, acetone and methyl alcohol.

*Preparation of 2:4:6:2':4':6'-hexaiodo diphenyl trichloroethane:*

In this condensation chloral (0.5 mol) was condensed with 2:4:6 triiodobenzene (1 mol) in the usual acid mixture at  $55-60^{\circ}\text{C}$  for 12-15 hours. After this period, the crude product was obtained in the usual manner by dilution with crushed ice and was dried in vacuo over calcium chloride. The crude material was first dissolved in alcohol under reflux and boiled for 30 minutes with the addition of charcoal, to remove colouring matters. The liquid was filtered hot and diluted with water when a faint yellow solid separated. This was filtered off and again dried in vacuo. The dry powder was then crystallised from benzene in light yellow needles. M.P.  $169-70^{\circ}\text{C}$ . The yield is 30% of theoretical.

*Properties.*—Insoluble in water, dilute acids hot and cold. Soluble with difficulty in hot alcohol, benzene, methyl alcohol and acetone. Easily soluble in xylene, chloroform, carbon tetrachloride and ether.

#### PHYSIOLOGICAL ACTION ON MALARIAL LARVAE

The three compounds were examined in 5% benzene solution and compared to a 5% benzene solution of DDT. The spreading power of the solutions were almost equal to that of a DDT solution (about 6 inches), but the activity of the compounds were found to diminish with increasing molecular weight. The hexachloro diphenyl trichloroethane has 75% of the activity of DDT on malarial larvae, while the corresponding bromo and iodo compounds possess 62% and 42% of the insecticidal property of DDT respectively.

Our best thanks are due to Dr. S. C. Niyogi, D.Sc. for his kind interest and advice and also to Prof. D. N. Roy, M.D., D.Sc., Professor of Entomology, School of Tropical Medicine, Calcutta, for the biological experiments.

NUTRITIONAL INVESTIGATION OF SUN DRIED FISHES  
AVAILABLE IN BENGAL.

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Fish constitutes one of the most important items of the daily diet of the people of Bengal. The reason no doubt is that Bengal delta is covered with a network of rivers and canals and a considerable supply of fish is available more or less throughout the year. During the seasonal gluts, a huge amount of fishes available and these fishes are preserved by the existing crude methods. The only known processing, practised in the province is sun drying and in some localities, salting and pickling. The total estimated product in Bengal including the Sylhet district is approximately 1225000 maunds annually. Thus sun dried fish is an important part of food and the nutritional values of these preserved fishes deserve investigation. In our previous communication, analysis of a few 12 sutki /sun

dried) fishes available in Calcutta market have been given. The present communication gives the result concerning the moisture, body fat, total mineral, protein, total iron, calcium, phosphorous, content of 15 varieties of sun dried fishes obtained from Contai curing centre.

### EXPERIMENTAL

The methods adopted in these investigations are the same as described in our previous papers (1-5). The results given in the tables are of edible portion only and are based on the analysis of 2-3 samples of each variety of fish. The zoological name of most of the fishes could not be procured.

TABLE I  
*Mean values of phosphorous, calcium, copper, iron (total) per 100 g. fishes.*

No.	Local name	Moisture per cent.	Body fat per cent.	Protein per cent.	Ash per cent.
1.	Halda Chingri	16.00	3.13	56.01	21.81
2.	Goga Chingri	15.00	—	55.44	21.28
3.	Sada Chingri	16.40	3.93	50.76	19.15
4.	Bali Kanakda	17.50	9.01	44.02	24.52
5.	Tapsi	16.20	12.08	58.46	17.20
6.	Rangi Chingri	17.80	—	56.32	22.89
7.	Pata machh	12.60	2.73	64.58	17.57
8.	Tangra	13.80	3.90	54.89	27.48
9.	Taltapra	11.00	16.34	54.52	16.76
10.	Chiki	14.58	6.30	54.69	24.20
11.	Rupapatar	14.20	14.25	54.55	15.50
12.	Modal machh	15.50	3.98	56.24	23.30
13.	Shankhachur	16.40	6.13	67.24	8.95
14.	Joyali magur	13.60	5.44	69.68	8.74
15.	Tapra	12.60	8.95	62.28	15.10

TABLE II  
*Mean values of phosphorus, calcium, copper, iron (total) per 100 g. fishes.*

No.	Local name	mg. Calcium	Phosphorous mg.	Copper mg.	Iron mg.
5.	Tapsi	1596.8	594.6	1.2	41.2
6.	Rangi Chingri	3846.7	827.6	1.4	46.9
7.	Pata machh	987.8	430.2	0.72	51.7
8.	Tangra	842.7	400.2	0.58	100.8
9.	Taltapra	906.8	412.6	0.82	88.7
10.	Chiki	1007.5	512.7	1.7	89.2
11.	Rupapatar	889.8	412.9	1.1	43.7
12.	Modal machh	548.9	448.2	0.9	22.0
13.	Shankhachur	779.7	500.7	0.76	11.9
14.	Joyali magur	1804.0	892.6	0.67	22.06
15.	Tapra	771.0	552.3	0.71	19.26

## DISCUSSION

In discussing the results a few points require clarification. Percentage of moisture of majority of samples varies between 12 to 17 ; moisture content of the sample no. 3 is as high as 30%. Optimum moisture percentage which stops bacterial growth is definitely below 12%. Thus it is evident that these sun dried fishes are not quite unsuitable a flora for bacterial growth and naturally these fishes are invariably infected, decreasing their keeping qualities. For complete elucidation of this point, bacterial examination of these sun dried fishes should be carried out.

The protein values of these fishes vary between 40 to 69% on moisture free basis. If we compare these results with those of the dehydrated fishes (protein values lying between 90 to 85% on moisture free basis) low protein content of the sun dried fishes becomes obvious. The low figure for protein results from the method of processing itself. In processing of sun dried fishes, evaporation of moisture is carried with the help of sun's radiation and as such the process of evaporation is naturally a slow one. Before the time when percentage of moisture has reached its lowest limit protein begins to decompose through internal enzymes and infection resulting in low value of protein for the final product.

The fat content varies widely from 2.03 to 14.25%. The figures for fat are not bad but the quality is definitely unwholesome giving rancid smell.

High figures for ash content, 8% to as much as 27.48% (Cf. ash content of dehydrated fish is between 3 to 5%) indicate likely contamination with sand and mud during the process of curing. Though the values for ash is high, the values for phosphorous, calcium, copper are within the range of normal values. Abnormally high values for iron is definitely due to contamination with mud and sand.

Thus, from every aspect, sun dried fishes are unsatisfactory for consumption. The reason underlying the production of such an inferior stuff lies in the process itself. Furthermore, the process of sun drying is not standardised at all. Considering the huge amount of sun dried fishes sold in the market and from the standpoint of hygiene and health the problem of preservation of fishes by drying requires investigation and improvement.

## SUMMARY

- (a) Moisture content of the sun dried fishes analysed, varies between 11.0-34.0%.
- (b) Body fat lies between 2.03-14.25%.
- (c) Protein content varies between 40.71-69.68%.
- (d) Values of ash ranges from 8.95-29.48%.
- (e) Calcium content lies between 548.9-3846.7 mg. per cent.
- (f) Phosphorus content lies between 400.2-827.6 mg. per cent.
- (g) Copper content lies between 0.58-1.70 mg. per cent.
- (h) Iron content lies between 100.8-11.9 mg. per cent.

## ACKNOWLEDGEMENT

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INVESTIGATION ON THE NEW ANTIDIABETIC PRINCIPLE  
(AMELLIN) OCCURRING IN NATURE

PART VIII. ITS ROLE IN THE DIRECT METABOLISM OF FAT  
IN THE EXPERIMENTAL ANIMALS (ALBINO RATS)

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Obesity has been regarded by Adams (1) and Joslin (2), as a precursor of diabetes in many cases, and Grafe (3) attributed it to the failure of the alleged metabolism stimulating mechanism in an individual. Several attempts have been made from time to time to find out means of reducing or rather checking adiposity.

Hetenyi (4) postulated that fat once deposited in the depots of an obese person is prevented from leaving them and so cannot be used as fuel. Work of Lauter (5) and Newbergh and his associates (6) have shown that energy required by an obese person to perform a given piece of work is more than that required by a normal subject.

The discovery of an antidiabetic principle "Amellin" and the clinical results (7, 8) obtained by its trial on diabetics and specially the observation that amellin can bring about reduction in hypercholesterolemia (9) and ketonuria (10) suggested the possibility that this might be helpful in bringing about direct metabolism of body fat in an individual.

The desaturation theory of Leathes and Wadell (11) has recently been confirmed by the work of Schoenheimer and Rittenberg (12) and so indication of increased fat metabolism through 'Amellin' may therefore be obtained not only by observing the check in the gain in weight of the animals receiving sufficient fat diet, but also from the estimation of 'the iodine number' of the Liver and body fat of the experimental animals and comparing them to those of the control ones.

•  
EXPERIMENTAL

A set of sixteen adult male rats were chosen for this experiment. These rats were divided into two groups. A and B, each group containing eight rats. (The rats of 'group A' were used as experimental animals, those of 'group B' being used as

controls). The rats were accommodated in four cages (labelled as cages I, II, III and IV), each cage containing four animals. Cages I and II contained animals of 'group A' while cages III and IV contained those of 'group B'.

As regards the body weight, the animals chosen were of two types, one type of animals weighing above 190 g. viz., those of cages I and III; the other type, weighing below 150 g. were contained in cages II and IV.

For about a fortnight before beginning the experiments, the rats were fed on a diet composed of whole wheat and milk; together with 0.5 c.c. of ghee per rat per day.

During this pre-experimental period, the rats were weighed regularly, every five days, and the average change in body weight noted.

After this period the rats in cages I and II were given by mouth a standard preparation (5 mg. per c.c.) of amellin at the rate of 1 cc. per rat per day, the diet being retained the same.

The Amellin was administered orally to the animals in the cages I and II in the morning together with a little milk. The control animals (cages III and IV) were given the same amount of milk at the same time. The main diet (whole wheat, milk and ghee) was given to the rats after an hour.

All the rats were weighed after every five days. The experiments were continued for about 80 days, after which all the rats were killed and the total body fat and the liver fat in each case were extracted with petroleum ether, in soxhlet apparatus.

The Iodine values of the body fats and liver fats so obtained were then determined, according to Hanu's method.

Table II gives the "Iodine values" so determined (each fig. in the table represents the average value for four rats in each cage).

## DISCUSSION

It is thus evident that amelin in daily dose of 25 to 50 mg. per kg. brings about such changes in fat metabolism, that the iodine numbers of the fats stored in the liver as well as in the whole system, becomes much higher than those in the controls. This observation confirms, according to Leathes and Wadell (*loc. cit*) how amellin participates in fat metabolism by conversion of saturated fatty acids into the more highly unsaturated ones, by a process of dehydrogenation and it is in this way that fats are rendered useful to the tissues, when the fatty acid chains can easily be broken up and oxidised.\*

Amellin, has previously been shown by Nath, Chakraborty and Banerjee (13) to possess some similarity in action with the haematopoietic principle of the liver. Here again we find, how, it resembles some liver constituent in bringing about dehydrogenation of fats too.

1. The effect of amellin in high dose in reducing excess of fat in adult rats has been studied.
2. Amellin has been shown to bring about further desaturation of the liver and body fat which has been known as an intermediate process in the metabolism of fat.

TABLE I

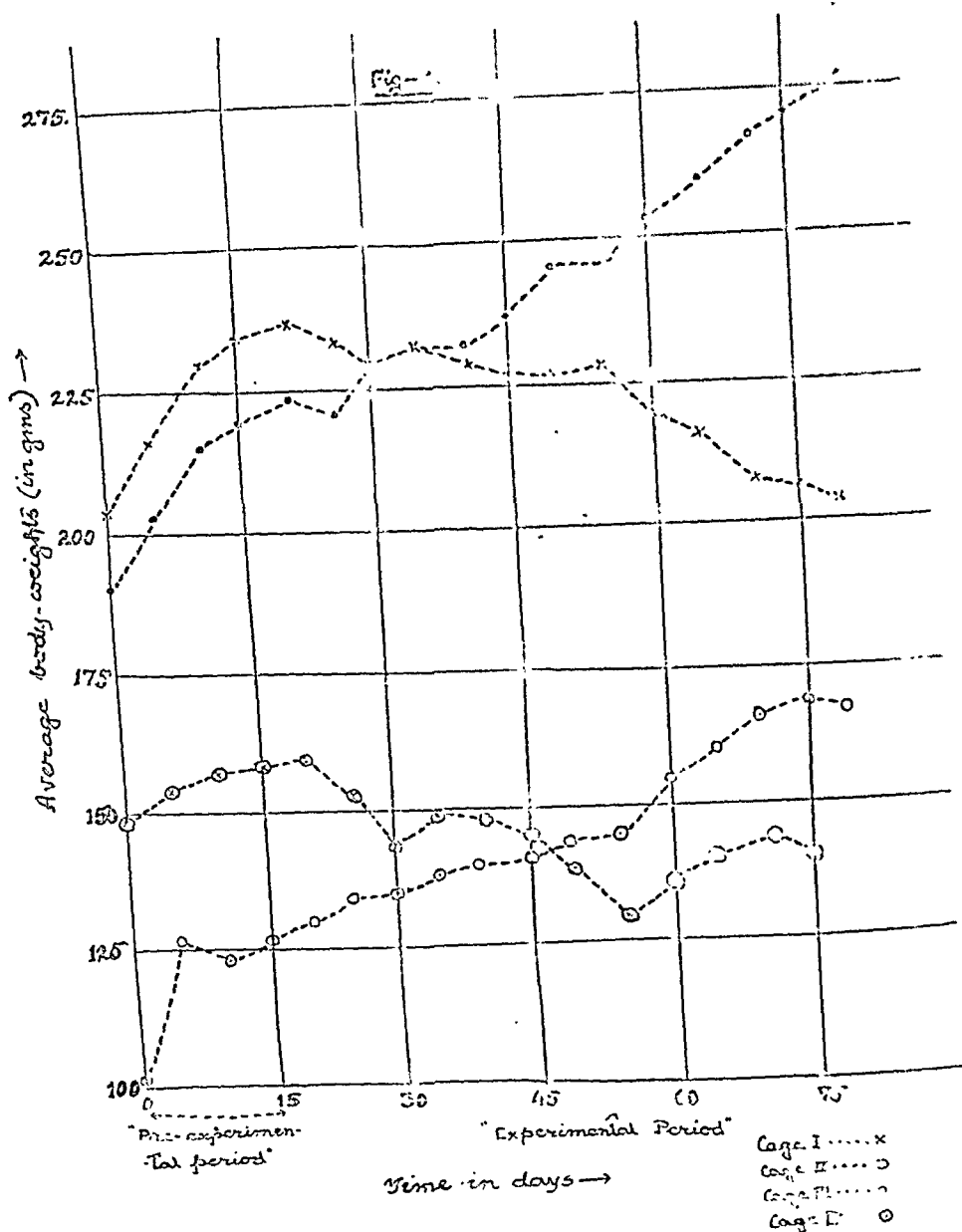
Case No.	Pre-experimental period Average body wts. in g.				in days.				Experimental period				Average body wts. in g.				in days.			
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80			
I	204	217	230	235	237	233	228	230	228	226	226	227	220	217	210	209	206			
II	149	154	157	158	159	152	144	149	149	145	140	130	136	141	144	138	138			
III (Control)	191	202	217	220	224	220	228	230	230	236	245	247	253	261	268	272	276			
IV (Control)	101	126	124	126	130	135	135	138	139	140	144	145	154	160	165	168	166			

Rats in the cages I &amp; II given amellin in the daily dose of 5 mgs.

TABLE II

Cage No.	Average wt. of liver fat per rat (g.)	Average wt. of body fat per rat (g.)	Iodine value of liver fat.	Iodine value of body fat.	Mean for 8 rats.	Mean for 8 rats.
I	0.18	4.8	63.4	47.3	62.15	43.9
II	0.15	3.2	60.9	40.5		
III	0.26	5.3	43.5	28.9	46.35	31.75
IV	0.11	2.9	49.2	34.6		





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\* In previous communications of this series, on the clinical observation on diabetics, only 2 to 3 mg. were used per kg. per day.

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## THE SERUM ESTERASE (BUTYRIC) ACTIVITY IN MALIGNANT DISEASE

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There have been very few investigations on the content of esterase in the tissues or in the serum in malignant disease and the significance of the findings is somewhat obscure. Falk, Noyes and Sugiura (1) used ten different esters such as ethyl or methyl acetates, butyrates and benzoates. They reported that the small absolute values for the enzyme actions of the tumour (Flexner-Jobling rat carcinoma) in comparison with the enzyme actions of many other tissues, were

striking. The heart, leg muscle and the brain actions are of the same order of magnitude but the actions of the other tissues are very much greater. They also found differences which depended upon the ester used ; it may be said that in general the actions were largest with the kidney and liver, that the testes followed closely and then the spleen and lungs. The magnitude of enzyme actions by the Flexner-Jobling rat carcinoma was however characteristic. Green (2) found that in rats during the growth of Jensen sarcoma the esterase activity of serum showed a pronounced reduction and then a parallel fall occurred in some of the tissues. The reduction in the serum esterase content started at an early stage of the tumor growth even when the animal appeared vigorous and healthy ; this ultimately reached a very low level. The significance of this large fall according to the author is, however, obscured by the fact that other unrelated conditions also produce a similar, though probably not so profound a change.

Cherry and Grandall (3) reported that there was no significant uniform change in blood esterase content as measured by hydrolysis of ethyl butyrate or tributyrin, following obstruction of the pancreas in dogs. Troeschler and Norris (4) used Wistar rats bearing implanted adenocarcinoma for the study of blood esterase. They showed that during the growth of the tumour, the esterase activity of the blood fell far below the range of fluctuation of the blood esterase values found for normal rats. In three of the rats which exhibited receding tumours the blood esterase activity rose towards normal values.

It was shown by Khanolkar and Chitre (5) that the esterase (butyric) activity in serum of a strain of highly inbred laboratory mice that are insusceptible to mammary cancer (C57), was almost the half the amount normally found in two susceptible strains (A and C<sub>3</sub>H). The livers of these strains were found to be equally potent as regard this enzyme activity (Chitre and Khanolkar, 6). The result of the excretion showed that the urinary excretion was almost equal but the, fecal excretion, and consequently the total excretion, was higher in the insusceptible strain than in the susceptible strains. No difference was noted in the kidney esterase. These results indicate that probably the livers of these strains do not differ in their capacity to elaborate the enzyme but do so in their ability to maintain an equivalent level in the blood. The effect of foster nursing on the esterase activity of serum and liver of a high mammary cancer strain (C<sub>3</sub>H) and a low mammary cancer strain (C57) was also studied, (Khanolkar and Chitre, 7). It was found that the reciprocal foster nursing resulted in a change in the serum esterase of the progeny which could be correlated with the susceptibility of the foster mother. Shimkin, Greenstein and Andervont (8) determined esterase activity in the serum of male and female mice of strains C.H. C57 Black, C, and I, and descendants of C mice that had been foster-nursed by C<sub>3</sub>H females. They however failed to correlate the esterase activity with the susceptibility to cancer. Greenstein (9) showed that the change in the esterase activity during the onset of tumour was variable depending upon the type and the site of tumour.

The above findings suggest that the esterase activity is somehow or the other related to malignant growth, but the exact significance remains obscure. It was, therefore, decided to study the same in certain types of human cancers. The breast cancer in human subjects was, therefore, chosen. In order to study whether the relationship, if any, is specific to cancer, it was necessary to extend the study to certain other diseases. These studies are reported presently.

## EXPERIMENTAL

## I. NORMAL SUBJECTS:

(a) *Males* : For the sake of normal standards 90 males between 30 and 40 years of age were chosen from the blood-donors of the hospital. Care was taken in choosing the persons who were in good health. The determination of the esterase concentration in serum was made by the method already described (Khanolkar and Chitre, *loc. cit.*). The range of the enzyme activity in the sera of these subjects was from 3.4 to 9.6, the average being  $5.60 \pm 0.14$ .

(b) *Females* : Female subjects of two age groups have been studied. They were chosen from the nursing staff of the hospital. They were studied nearly a week past their periods. The subjects chosen were in good health. The first age group was from 20 to 35 years, while the second was 35 to 60 years. In the former group of 24 subjects the range of serum esterase was from 4.6 to 8.6, the average being  $6.12 \pm 0.26$ ; while in the latter group of 16 subjects the range was from 4.2 to 6.9 with an average of  $5.10 \pm 0.20$ .

## 2. DISEASES:

(a) *Leprosy* : 25 male patients with leprosy from Ackworth Leper Home, Mātunga, with positive smears (positive acid fast bacilli) whose age varied from 16 to 50 years were studied for their serum esterase concentration. The range of the enzyme activity was from 1.6 to 4.5, the average being  $3.14 \pm 0.11$ . There were also 20 patients with negative smears and with the age between 18 to 53 years; they had the range from 1.7 to 5.7, the average being  $3.64 \pm 0.21$ .

(b) *Syphilis* : 14 patients from Sir J. J. Hospital, suffering from Syphilis were studied for their serum esterase content. The range amongst them was found to be from 2.1 to 4.1 with the average of  $3.6 \pm 0.15$ .

(c) *Tuberculosis* : 12 females suffering from tuberculosis from Turner's Tuberculosis Sanatorium were studied. Their age varied from 17 to 36 years. The range of serum esterase was found to be from 2.1 to 4.5, the average being  $3.47 \pm 0.20$ .

(d) *New Growth* : (i) Malignant (cancer of the breast):

32 females suffering from the carcinoma of the breast, as established by the histological examinations, were studied. Their ages varied from 35 to 68 years. The range of serum esterase was found to be from 1.9 to 4.8 with the average of  $3.67 \pm 0.14$ . These cases were chosen from the patients admitted to the hospital and estimations were done prior to any treatment.

## (ii) PHYSIOLOGICAL GROWTH (PREGNANCY):

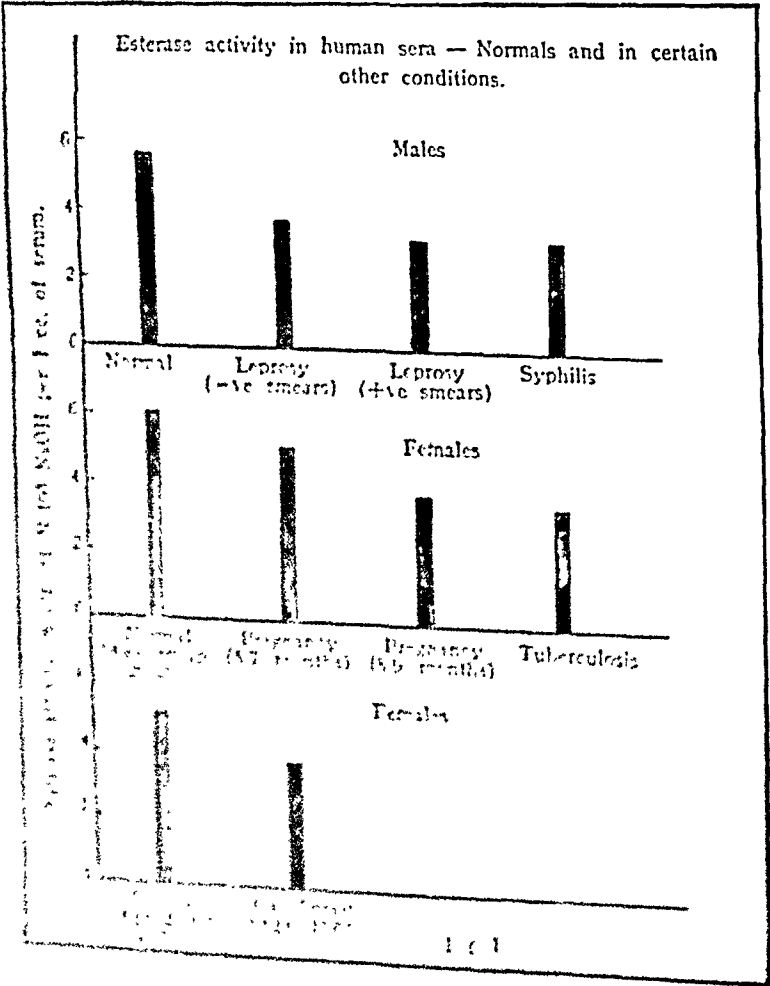
Two groups of pregnancy cases were studied. They belonged to the Maternity Hospital in the city. The first group consisted of the subjects of 5 to 7 months of pregnancy and the second of 8 to 9 months. In the former group of 18 cases the age varied from 17 to 30 years. The range of serum esterase was from 4.4 to 5.7, the average being  $5.07 \pm 0.10$ . In the latter group of 10 cases, the age ranged from 20 to 30 years. The range of serum esterase was found to be from 3.4 to 4.2 with the average of  $3.72 \pm 0.03$ .

In Table No. 1 are shown the mean results of esterase activity in normal and in various diseases.

TABLE I

*Esterase activity in normal and in various diseases.*

Subject	Normals		Febrile Diseases				New Growth			
	Males	Females	Leprosy	Syphilis	Tuber- culosis	Ca- Breast	Pregnancy			
	Age group 25-35 Years	Age group 35-60 Years	Posi- tive smears	Nega- tive smears			5-7 mos.	8-9 mos.		
Number	(20)	(24)	(16)	(25)	(20)	(14)	(12)	(32)	(18)	(10)
Maximum	9.6	8.5	6.9	4.5	5.7	4.1	4.5	4.8	5.7	4.2
Minimum	3.4	4.6	4.2	1.4	1.9	2.1	2.1	1.9	4.4	3.4
Mean	5.66	6.12	5.10	3.14	3.64	3.16	3.47	3.67	5.07	3.72
St. Error	±0.14	±0.26	±0.20	±0.11	±0.21	±0.15	±0.20	±0.14	±0.10	±0.03



## DISCUSSION:

The result of investigations at the hands of various workers on the content of esterase in serum in malignant disease are not decisive in arriving at a definite conclusion with regard to the change in the activity of the enzyme during malignancy in different species. Thus the work of Green (2) and Troescher and Norris (4) showed that the enzyme behaved differently in tumour growth in case of animal such as rats and in case of human subjects. These findings, however, suggested that the changes in esterase activity in serum are probably an expression of some metabolic disturbance in the body which accompanied the growth of the malignant tumour and that it forms a part in the function of the abnormal physiology associated with the presence of cancerous growth. References could also be found in the literature that there exists more or less a similarity with regard to the behaviour of some enzymes in cancer and in infectious diseases such as tuberculosis or even the condition of physiological new growths (such as pregnancy). Thus the arginase content of liver and spleen decreased rapidly following tumour transplantations to the animals and a similar phenomenon occurred in tuberculous animals (Fujiwara, 10). An increased nucleolytic activity has been reported for sera of tumour patients and similar findings were obtained in tuberculous patients (Wolter, 11). The atoxyl-resistant lipase frequently disappears from the serum in advanced stages of the tumour process, while it may also be encountered in the presence of increased quantities of newly formed epithelial elements, such as in regenerative process or in pregnancy (Bernhard, 12). For the present study the carcinoma of the breast was chosen since it has been reported that there was probably some relationship between the susceptibility of animals to mammary cancer and the activity of this enzyme in the blood (Khanolker and Chitre, *loc. cit.*). With regard to the chronic infective diseases, the patients suffering from leprosy, tuberculosis and syphilis were studied. Cases of pregnancy between 5 and 7 months and between 8 and 9 months were also included. The objects in view in choosing such variety subjects for study were threefold

Firstly, to see whether the condition of the general breakdown of the normal functions, which commonly occurs in cancer and in infectious diseases like tuberculosis etc. is similarly reflected in the changes in the activity of the enzymes in these diseases. Secondly, to see whether the requirements necessary for growth (both malignant and physiological) are identical in respect to the enzyme under study. And that both types of growth influence the metabolism in similar manner. The researches of Weil and Russel (13) on proteinase give support to such a surmise. For this purpose normal pregnancy could be compared with cancer growth. And thirdly, the enzyme which was taken for study was lipase and as such might be supposed to play an important role in fat metabolism. Its study, therefore, in different conditions, such as tuberculosis and pregnancy, and its relation with cancer might give some information as to the effect of cancer on fat metabolism.

From the table it could be seen of normal subjects that there is no significant difference in males and females as regards the enzyme activity in serum. A slight effect, due to age, is observed in the two age groups of female subjects studied. Thus, in the subjects belonging to the age group of 35 to 50 years, the enzyme activity in serum is at a slightly lower level than that seen in the age group of 20 to 35 years. It has been reported however, by Thompson and Whittaker (14),

during the study of esterase of skin that the tributyrin activity somewhat increases and the methylbutyrate activity markedly increases with age. In our experiments ethyl butyrate has been used as the substrate and it is very likely that this might cause any difference.

As regards the concentration of the enzyme in serum in various infectious diseases, there appears to be a general tendency for the activity to decrease. The lowering of the enzyme level in the diseases (namely tuberculosis, syphilis and leprosy) is almost to the same extent. In case of leprosy, the activity of the enzyme is at a slightly higher level in the patients with negative smears than that in the patients with positive smears, although difference is not statistically significant. In patients with carcinoma of the breast there is significant lowering of the enzyme activity in serum if compared with the activity in the corresponding normal subjects. If this extent of the decrease is compared with that found in other infective diseases, there is marked difference between the two conditions. In pregnancy also the level of the enzyme activity in serum has gone down in comparison with that observed in the normal subjects of similar age. However, the effect is not so marked in cases of 5 to 7 months of pregnancy. It is well marked when the pregnancy has sufficiently advanced to the period of 8 to 9 months. This lowering, however, is of the same order as is seen in either cancer of the breast or other diseases.

These findings are more or less in agreement with the findings of other workers on various enzymes. Thus, the activity of the serum lipase has been found markedly lower in some cancer patients and similar findings were also observed in advanced tuberculosis (Bauer, 15). Similarly the serum esterase was found below normal level in the terminal stage of the cancer in human patients, by Green (*loc.cit.*). Albers (16) found phosphatase activity of sera of tumour patients increased similar to that in tuberculosis. Weil and Russel (*loc.cit.*) studied the plasma proteinase activity in rats with Philadelphia No. 1 sarcoma, Jensen sarcoma and Walker No. 256 carcinoma and in pregnancy of rats and found that the activity dropped considerably during tumour growth as well as in pregnancy. When the tumour was removed by operation or after termination of pregnancy the enzyme activity rapidly returned to the normal level. The authors, therefore, conclude that the similarity in results obtained during the tumour growth and in pregnancy indicated that the observed phenomenon is in some way connected with rapid tissue growth. This relationship is emphasized by the rapid return to normal values of proteinase activity after termination of the causative factor (tumour removal or terminate of pregnancy).

An attempt can also be made here to explain the decrease of the esterase activity in serum in various conditions studied, on the basis of the fat metabolism that might be assumed to be undergoing change in some way or other in these conditions. One of the oldest references to fat metabolism in neoplasia may be found in Freund's theory (Freund and Kaminer, 17), which regards processes of fat digestion as being a decisive factor of development and growth of tumours. Some support for the assumption of a disturbed intermediate fat metabolism in neoplasia may be obtained in the work of Gaessler (18). The author states that whereas, fat intake normally causes an increase of ketone bodies in the blood, particularly of  $\beta$ -hydroxybutyric acid, this effect was found to be absent in cancer patients that is the concentration of ketone bodies in the blood failed to increase following

the ingestion of fats. Some additional evidence for an alteration of intermediate fat oxidation may be derived from analytical findings. Thus it has been stated that fatty acids contained in the blood of cancer patients possess a higher molecular weight than those present in normal blood. This increased molecular weight may be interpreted as being a consequence of a disturbed chain-reduction of the fatty acids, i.e.  $\beta$ -oxidation (Pourbaix and Buytaert, 19). Furthermore, the observation of increased concentrations of unsaturated fatty acids in the blood of cancer patients may indicate an interruption of the fat oxidation after dehydrogenation (Ascoli, 20). Studies *in vitro* give support to the alterations in the fat metabolism in malignant tumours. Slices of human neoplasm, of Ehrlich mouse carcinoma and of Rous fowl sarcoma were found incapable of oxidation monocarboxylic fatty acids (saturated or unsaturated); sometimes even a reduction of the oxygen consumption was observed after the addition of fatty acids. The  $\beta$ -oxidation of fatty acids in neoplastic tissue takes place in the same manner as in normal tissues, whereas the  $\beta$ -oxidation was found absent (Ciaranfi, 21). Thus, all these investigations go to suggest that the fat metabolism is in some way or other altered in neoplasia and it may be imagined that the activity of the enzyme under discussion, might undergo change in relation to this altered fat metabolism. It should, however, be mentioned the argument supporting the change of fat metabolism so as to affect the esterase activity need further support by experimentation. Till then the statement is one among many possibilities in tumour growth.

#### SUMMARY AND CONCLUSION:

The esterase activity in serum is at a lower level, in comparison with the normals, in infective diseases such as leprosy, tuberculosis and syphilis, in carcinoma of the breast and in later pregnancy. The decrease in these various conditions is approximately the same.

It is suggested that the changes observed in cancer, pregnancy and other diseases might probably be due to altered fat metabolism.

#### ACKNOWLEDGEMENT

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## HYPOPROTEINEMIA AND OEDEMA

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A study of serum protein concentration in cases of oedema is increasing in importance in order to plan a proper therapy. Hypoproteinemia is mostly due to the fall of the albumin fraction of the serum protein. Albumin has a great influence in the maintenance of the blood volume and the water balance in the body due to its greater osmotic influence. The knowledge of this fact is of recent origin, and actual period of investigation on serum protein began when Epstein (1) observed reserved A/G ratio in nephrosis. Particular mention may be made to the work of Moor and Van Slyke (2) Benjamin and Kagan (3, 4)

The present investigation is a study of protein, albumin and globulin in serum of 130 cases of oedema. Attempt has been made to establish a relationship between the albumin and globulin fraction in order to find out if it can serve a useful laboratory guide in establishing the aetiology of oedema and whether it is advisable to give protein value alone or the absolute values of albumin, globulin and A/G ratio are essential.

### METHOD

Total nitrogen of the serum was determined by the micro-kjeldahl method using methyl red as indicator and the total proteins were calculated by multiplying the total nitrogen thus found by the factor 6.25. This study was undertaken with a view to helping the clinical investigation. Non-protein nitrogen was not estimated but an allowance of protein equivalent to 10 mg. of nitrogen was made. The albumin was determined after precipitating the globulin fraction from the serum with 22 per cent sodium sulphate at 38° in an incubator for 4 hours or over night.

## RESULTS

Indoor patients with oedema were selected for this study. The differential diagnosis was made by the visiting physicians based on the finding of their physical examinations and laboratory reports. These cases have been arranged into three groups (i) serum protein below 5.5 per cent, (ii) protein between 5.5 and 7.5 per cent and (iii) protein above 7.5 per cent.

In Table I are shown group by group the mean concentration of serum protein, albumin and A/G ratio. Albumin and A/G ratio in these groups gave a high coefficient of variation and covered a range of values overlapping each other in groups and among groups. This being so it is difficult to predict the albumin or A/G ratio from a given value of a serum protein.

TABLE I

*Correlation between protein, albumin and A/G ratio.*

	Group I	Group II	Group III	Combined Group
<i>Serum protein per cent.</i>				
Range	Less than 5.5	5.5 to 7.5	More than 7.5	3.06 to 9.85
Mean	4.77	6.31	8.07	5.85
Coefficient of variation	22.4	11.2	10.1	12.7
<i>Albumin per cent.</i>				
Range	0.87 to 4.00	1.09 to 4.72	2.90 to 5.90	0.87 to 5.90
Mean	2.06	3.08	4.43	2.77
Coefficient of variation	34.4	28.9	23.7	40.0
<i>A/G ratio</i>				
Range	0.30 to 2.75	0.20 to 2.95	0.43 to 2.13	0.20 to 2.95
Mean	0.82	1.03	0.58	0.97
Coefficient of variation	59.0	53.4	43.2	55.0

Table II shows that out of the 130 cases of oedema 55 cases (42 per cent) had serum protein less than 5.5 per cent, 61 cases (47 per cent) had serum protein between 5.5 and 7.5 per cent and 14 cases (11 per cent) had serum protein above the normal limit of 7.5 per cent. Eighty five per cent of these cases had albumin concentration less than 4 per cent. This lowering of albumin concentration is marked in groups with low protein concentration. This is an indication that hypoproteinemia develops at greater expense to serum albumin.

TABLE II

*Distribution of total protein and albumin in serum.*

Total protein % Albumin %	Less than 5%	5.5 to 7.5%	More than 7.5%	Total	Percentage
More than 4.0%	1	8	10	19	14.6
2.0 to 4.0%	27	49	4	80	61.6
Less than 2.0%	27	4	0	31	23.8
Total	55	61	14	130	—
Percentage	42	47	11	—	—

Chi-square for Table II was found to be 72.5. This value when referred to Fisher's table for four degree of freedom gave a probability figure less than 0.01, indicating that the lowering of protein in hypoproteinemia is statistically significant and is highly so.

TABLE III

*Distribution of total protein and globulin in serum.*

Total protein % Globulin %	Less than 5.5	5.5 to 7.5	More than 7.5	Total	Percentage
More than 3	21	44	9	74	57
1.5 to 3	32	17	5	54	41
Less than 1.5	2	0	0	2	2
Total	55	61	14	130	—
Percentage	42	47	11	—	—

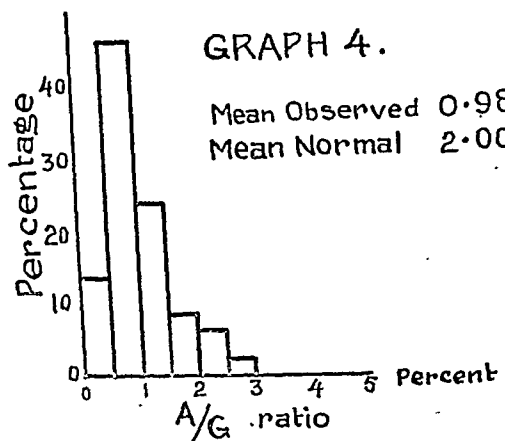
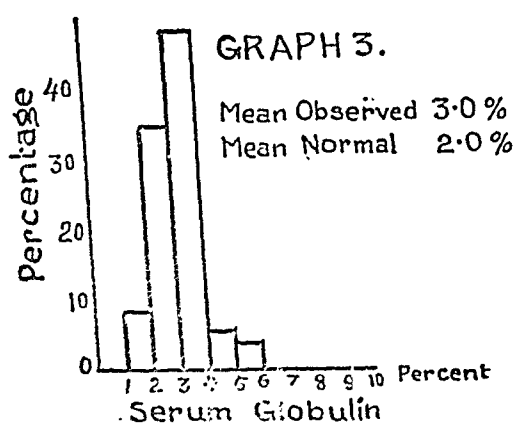
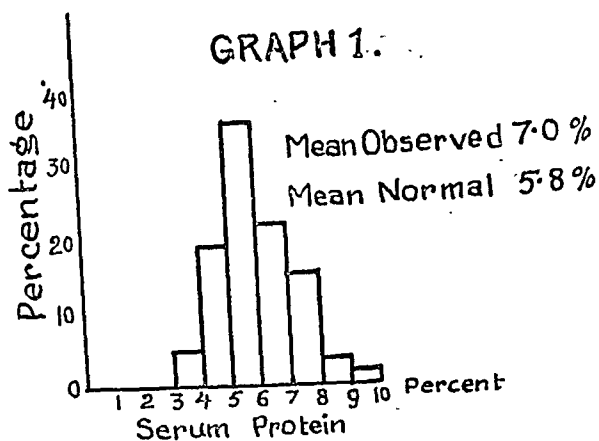
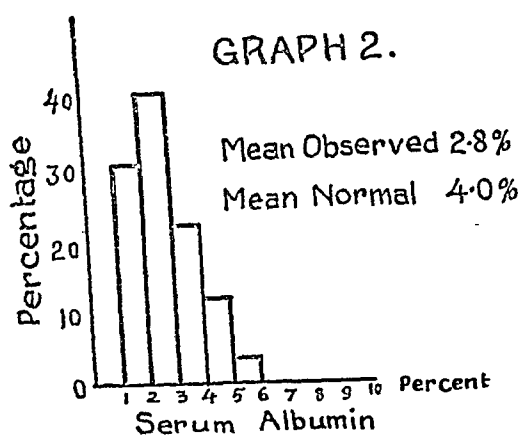
The serum globulin concentration (Table III) was higher than its normal limit of 3 per cent in 57 per cent of the cases. Unlike albumin, these high values of serum globulin were distributed at random amongst the three groups but the low values were confined mostly to the first group (serum protein less than 5.5 per cent).

TABLE IV

*Distribution of total protein and A/G ratio.*

Total protein % A/G ratio	Less than 5.5	5.5 to 7.5	More than 7.5	Total	Percentage
2 to 3	3	6	2	11	8.5
1 to 3	12	22	8	42	32.3
Less than 1	40	33	4	77	59.2
Total	55	61	14	130	—
Percentage	42	47	11	—	—

Table IV indicates that 91.5 per cent of the oedema cases had A/G ratio lower than its normal value of 2. This lowering of A/G ratio was not confined to any particular cell but was distributed equally in all the three groups showing absence of any correlation between A/G ratio and serum protein.



Graphs 1 to 4 indicate that in the combined group there was a slight lowering in serum protein, marked decrease in serum albumin, a general increase in globulin and a wide deviation and inversion of the A/G ratio.

In Table V the observations are arranged with respect to disease. It is presumed that these different samples have been drawn from a universe comprising of various diseases which differ in the variability of their individuals. The difference between the means (combined group and the disease group) that differs by more than twice the square root of the sum of the squares of their standard errors  $\frac{m-m}{e_1^2 + e_2^2}$  were relatively rare. These values did not give sufficient evidence to lead to the conclusion that the differences are more than what might be due to chance i.e., differences were insignificant. In cases of anaemia this value was 10; it is, therefore, likely that the samples in this group differed markedly from the combined disease group. In cases where the aetiology of oedema was other than diseases associated with anaemia such as nephritis, heart disease etc. the fall in serum protein concentration was comparatively less. Though the mean values of the serum

TABLE V

Serum proteins in diseases associated with oedema.

Disease	No of cases	Protein %				Albumin %				A/G			
		Range	Average	Coefficient of variation	$\frac{m_1 - m_2}{\sqrt{e_1^2 + e_2^2}}$	Significant	Range	Average	Coefficient of variation	$\frac{m_1 - m_2}{\sqrt{e_1^2 + e_2^2}}$	Significant	Range	Average
Nephritis including nephrosis	54	3.77-8.34	5.61	17.3	1.6	no	1.31-4.85	2.62	33.7	1.6	no	0.37-2.7	0.92
Anemia with nephritis	9	3.77-6.02	5.21	12.6	2.9	yes	1.31-3.50	2.20	34.1	2.1	yes	0.46-1.16	0.73
Heart cum nephritis	9	4.81-7.87	5.95	26.7	0.1	no	1.75-4.37	2.92	30.5	0.5	no	0.54-2.00	1.00
Anemia	22	3.06-7.65	3.33	35.1	1.0	yes	1.13-4.90	2.71	43.2	0.2	no	0.37-2.95	1.15
Heart	14	4.50-7.96	6.22	17.5	0.1	no	1.75-5.41	2.30	52.1	1.4	no	0.46-2.60	0.94
Liver cirrhosis	8	5.08-7.41	5.92	14.2	0.2	no	1.14-3.68	2.68	31.3	0.3	no	0.33-1.23	0.86
Dysentery	7	5.25-7.27	5.49	18.2	1.3	no	1.70-4.47	2.69	30.1	0.2	no	0.66-1.17	0.91
Combined disease group	130	3.06-9.85	5.85	12.7	—	—	0.87-5.59	2.77	40.1	—	—	0.20-2.72	0.98

Note:  $m_1, m_2$ , Differences between the mean of the combined group and the disease group.

$e_1^2 + e_2^2$ , Sum of the squares of the standard error of the combined group and the disease group.

protein in oedema associated with anaemia was lower than the values in diseases dissociated with anaemia, the individual values covered a range which over-lapped each other. A general decrease in the serum albumin was noticed in these groups but the serum protein was markedly less only in anaemia. May be that the serum globulin was invariably increased in diseases associated with oedema, in 57 per cent of the cases it was found to be higher than 3 per cent and thus the lowering or reversing of A/G ratio was a frequent event (91.5 per cent). No attempt, however, has been made to find out the cause (whether due to infection or some other cause) of the increase in the globulin fraction.

### SUMMARY

1. Hypoproteinemia (less than 5.5 per cent of serum portion) was shown by 42% of the cases of oedema, whereas hyperproteinemia (more than 7.5 per cent of serum protein) was present in 11 per cent of cases. Invariably the globulin content was in the upper limits of normal value or slightly raised. Hypoproteinemia was, thus, developed at a greater expense to serum albumin.

2. The fall in serum protein in oedema was maximum in diseases associated with anaemia.

3. There exists no quantitative relationship between the serum protein and A/G ratio in oedema. It seems essential to know the absolute values of serum protein, albumin and A/G ratio in order to draw any conclusion about the aetiology of a disease.

### ACKNOWLEDGEMENT

I am grateful to Dr. Swaroop Ph.D., for his help in obtaining the statistic values.

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**GLYCOGEN STORAGE AND LACTIC ACID OXIDATION AS AFFECTED BY  
DIFFERENT HIGH CARBOHYDRATE DIETS WITH AND  
WITHOUT AMELLIN**

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Attempts have hitherto been made by several workers from time to time to ascertain the aetiological factors causing diabetes mellitus. Harmful effect of high carbohydrate diet (71.3% approx.) has been recorded in case of monkeys by Radhakrishna Rao (1) who observed atrophic changes in the small intestine and fatty





## EXPERIMENTAL

## PART I.

18 growing albino rats were divided into 3 groups of six in each and three from each of these groups were put in a separate cage and the average values of the experimental results obtained were taken to represent that group.

*The stock diet consisted of*

Corn starch	...	...	...	470 g.
Casein	...	...	...	120 g.
Butter fat	...	...	...	40 g.
*Salt mixture	...	...	..	30 g.
Cod liver oil	...	...	...	20 g.
Marmite	...	...	..	20 g.
				<hr/>
				700 g.

30 g. of different types of carbohydrates i.e., starch, cane sugar and glucose were mixed with 70 g. of this stock diet thus making the percentage of carbohydrate 77 in all diets. Thus the animals under different groups received the diets as follows:—

Group A on Diet No. 1	containing 77% Starch
Group B on Diet No. 2	.. 47% starch + 30% sucrose
Group C on Diet No. 3	.. 47% starch - 30% glucose.

The feeding experiment lasted for 5 weeks and the weights of the animals during the experimental period are shown in table I. The animals were then killed by sudden strike on the head and the report of the autopsy findings as regards the condition of the different organs of the animals is represented in table II. Immediately after killing the animals, the liver and muscle were removed and glycogen and lactic acid contents were estimated according to the method mentioned below. The results are shown in the table III.

## PART II.

12 growing albino rats of almost the same age and body wt. as in (A) were selected for studying the additional influence of amellin (.5 mg. per 100 gm. of body wt.) on different types of high carbohydrate diets as described above. They were kept in 6 cages two in each and two pairs of animals were kept on each type of the experimental diet. These results are shown in Table IV.

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\* McCollum Davis salt mixture was used.

*Estimation of Glycogen.* Glycogen is estimated according to the method of Flyger as modified by Good, Kramer and Somogyi (13) which comprises of:

- (a) Alkali hydrolysis for 10—20 minutes.
- (b) Precipitation of glycogen with 95% alcohol.
- (c) Hydrolysis of glycogen to glucose.
- (d) The determination of glucose by the method of Shaffer and Hartmann (14)

*Estimation of Lactic Acid.* Lactic acid was estimated according to the method of Cori and Cori (15) and the method comprises of:—

- (a) Precipitation of protein with mercuric chloride,
- (b) Removal of glucose from protein free filtrate with copper sulphate and calcium hydroxide.
- (c) Conversion of lactic acid to acetaldehyde which is then combined with sodium-bi-sulphate.
- (d) determination of bound bi-sulphate idometrically.

#### *Average normal values of liver and muscle glycogen*

The normal values of glycogen have been found to be distributed within a wide limit by different groups of workers. According to Karezag, MacLeod and Orr (16) and Barbour, Chaikoff, MacLeod and Orr (17) the average value for 24 rats in separate sets of experiments is 160 mg. per 100 gram of the tissue. While this was found to be 90 mg. and 50 mg. by Carton and Lewis (18) and Silberman and Lewis (19) respectively. The results of Eckstein (20) however, show great variation ranging from 60-100 mgs. Results far exceeding these values have also been recorded by some investigators. The authors therefore thought it desirable to find out the normal values with 8 rats kept on laboratory normal diet and the average values for the liver and muscle glycogen were found to be 167 mg. and 136 mg. per 100 g. of tissues respectively.

#### DISCUSSION

Addition of glucose to the high starch diet has been found to bring about a beneficial effect to the young albino rats in various ways ; while cane sugar has been shown to bring about detrimental effect as with high starch diet alone, the percentage of carbohydrates, proteins and fats being retained the same in all the cases. It will be shown from table I that high starch + glucose diet, though inadequate for proper growth, has much better effect towards growth and maintenance than that with the high starch + sugar or starch alone, in case of growing rats. It was found on autopsy that the condition of the pancreas was almost healthy in case of animals receiving high glucose diet. While pancreas of those with high starch and starch plus cane sugar diet were generally affected, partial degeneration being observed in some cases.

TABLE I

Showing the average change of body weight of the rats in g.

[illegible]



TABLE III  
*Showing the change in glycogen and lactic acid content of the liver and muscle  
 in mg. per cent (average).*

Group	Cage No.	Nature of diet.	Rat No.	Glycogen.			Lactic Acid.				
				Liver	(means)	Muscles	(means)	Liver	(means)	Muscles	(means)
A	X	Stock diet + Starch		146.0	150.0	134.0	136.0	580.0	467.5	300.0	305.0
	XI			154.0		138.0		415.0		310.0	
B	XIV	Stock diet + Cane sugar		128.0	139.0	142.0	138.0	400.0	415.0	315.0	307.5
	XV			150.0		134.0		430.0		300.0	
C	XVIII	Stock diet + Glucose		159.0	177.0	150.0	156.0	170.0	184.5	130.0	135.0
	XIX			195.0		167.0		199.0		140.0	

TABLE IV  
*Showing the average glycogen and lactic acid contents of the liver and Muscle  
 in mg. per cent.*

Group	Nature of diet.	Cage No.	Rat No.	Initial body wt.	Glycogen in mg. per cent.			Lactic acid in mg. per cent.		
					Liver	(means)	Muscle	Liver	(means)	Muscle
A	Stock diet + Starch + Amellin	XX	113	98	195	199	150	215.0	198	130.0
			121				120			160.0
		XXIII	114	101	202			180.0		
			122							
B	Stock diet + Cane sugar + Amellin	XXI	109	96	190	195	140	205.0	193	130.0
			123				150			150.0
		XXIV	110	100	200			180.0		
			124							
C	Stock diet + Glucose + Amellin	XXII	105	97	180	188	145	150.0	165	140.0
			125				160			161.0
		XXV	106	106	195			180.0		
			126							

Regarding storage and depletion of the glycogen in the liver and muscle, it can be said that cane sugar diet caused marked depletion of this substance while glucose diet, though showing no excess storage, gives more or less similar results as with normal carbohydrate diet.

Increased amount of Lactic acid content of the liver and muscle, in case of animals kept on high starch supplemented with cane sugar, points out how indolent habits of taking these things in excess may cause symptoms of acidosis in the long run. Almost normal values of Lactic acid in case of animals given starch + glucose, furnishes at the same time some clue how additional glucose may cause better metabolism and how its increase in concentration in the blood shows but a physiological response to combat other abnormalities caused by accumulation of several intermediately metabolism products in the blood as observed by Nath and Brahmachari (loc. cit.).

The effect of amellin on the high starch and high cane sugar diet is worth noting. It will be evident from table IV that amellin can not only help storage of both liver and muscle glycogen but can also help in the disappearance of the high amount of lactic acid from the liver and the muscle which was found to be accumulated through the intake of high starch and cane sugar diet. This also explains well how amellin could cause relief in acidoses and could increase in alkali reserve of the diabetics as reported by Nath, Chakraborty, and Brahmachari earlier (21).

#### SUMMARY

1. Effect of different types of high carbohydrate diet (starch, starch + sucrose, starch + glucose) on growing albino rats has been studied.
2. High starch diet (77% starch) as well as the stock diet (47% starch) supplemented with 30% cane sugar caused marked retardation of growth of growing albino rats, while addition of 30% glucose to the stock diet showed marked better effect as far as the growth is concerned.
3. Cane sugar diet caused depletion of liver and muscle glycogen while glucose diet has no such evil effect.
4. Lactic acid contents of the liver and muscle far exceeded the normal limit in the animals receiving high starch and starch + cane sugar while those of rats receiving starch + glucose showed more or less normal value.
5. Addition of amellin to these high carbohydrate diets in the dose of 5 mg. per 100 gms. of body wt. can show high beneficial influence in checking the depletion of glycogen storage and in reducing the high percentage of lactic acid in the liver and the muscle.



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THE ROLE OF AUTONOMIC NERVES IN THE CONTROL OF THE  
MECHANISM OF CIRCULATION IN THE SYSTEMIC BLOOD  
VESSELS OF THE FROG\*

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The Frog has long continued to be a valuable aid for the realisation of the complex physiology involved in the various functional processes of the human body : unfortunately, very little is known as yet about the mechanism which regulates the flow of blood through the systemic vessels of the animal.

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\* An earlier report on this work was sent to the Indian Science Congress, Bangalore, 1946.

Observers, at different times in the past, noted the effects of various physical and therapeutic agents on these vessels. Adler (1) recorded the effect of perfusion of the lung and skin vessels of the amphibian. Amsler and Pick (2) noted their impression about the splanchnic area and limb vessels after similar experiments. Pearce (3) found that frog's blood vessels did not dilate with dilute solutions of epinephrin or with increase of temperature ; they dilated, however, in the absence of calcium or with the degeneration of their nerves. Kolm and Pick (4) found that acetyl cholin, like adrenalin, constricted frog's blood vessels when perfused alone ; perfused together, however, they caused dilatation. Hildebrandt (5) observed that atropin antidoted epinephrin and sympathetic nerve stimulation. Luckhardt and Carlson (6) were of opinion that there was an unusual innervation of frog's blood vessels. Cori (7), later Langecker (8), pointed out that there was seasonal variation in the vagus response of the frog which diminished markedly during summer. Cori attributed this to thyroid changes in the animal, and not to any altered potassium and calcium ratio, which, under certain conditions, diminished parasympathetic response (Burridge, 9):

For the first time, Vimtrup (10) made an attempt to explain the mechanism of blood flow through the smaller vessels of the animal, and reported contraction of Rouget cells which caused folding of the endothelium. Zweifach (11) found that mechanical stimulation caused contraction of the endothelium in the frog which blocked the lumen of the vessel at where the capillary left the arteriole. Field (12), also working with the frog, confirmed the findings of Vimtrup, and further observed that swelling of the endothelial nuclei blocked the lumen of the capillary. Clark and Clark (13) reported endothelium to be contractile in the tadpole's tail ; but they could not confirm Vimtrup's findings except for the nictitating and hyaloid membranes of the frog.

It would thus appear that observers differed widely from time to time in their appreciation of the exact mechanism which remained unrevealed till Fulton and Lutz (14) examined, "by means of stimulation with a micro-electrode, the distribution of the contractile elements of the small blood vessels in the retrolingual membrane of the frog," brain and medulla pithed. They concluded from their observations that in the retrolingual membrane of the animal, the capillary origins were provided with modified smooth muscle cells, of the nature of Rouget cells, in contrast to the uniform layer of typical smooth muscle of the arteriole, and the somewhat scattered arrangement on the precapillary ; these regions of capillary origin frequently acted in the manner of a sphincter independently of the supplying vessels and thus regulated capillary blood flow ; occasionally, they also acted as a unit with the adjacent blood vessels. As regards the nervous mechanism of control, they obtained histological evidences which revealed the existence of a perivascular, loosely meshed, non-myelinated nerve plexus which appeared to be anatomically continuous ; it was rich on the arterioles and the precapillaries, but sparse on the capillaries. "Faradic stimulation of the small nerves there produced responses confined to limited vascular areas ; therefore, though the plexus appeared to be anatomically continuous, functional innervation was discontinuous. Stimulation of any one of

the small nerves always produced a response in the same limited area, suggesting thereby a smooth muscle motor-unit." The response was either entirely dilator in nature, or constrictor, or both mixed. "Brief Faradic stimulation of the small vasomotor nerves produced dilatation of the small blood vessels, followed by constriction. Weak stimulation usually produced only dilatation. Strong stimulation of the same nerve frequently produced only constriction. These results suggested that the nerves stimulated contained both vasodilator and vasoconstrictor fibers, and that the vasodilators possessed a lower threshold. Vasomotor nerves were also found which produced only one type of response to all strengths of stimulation." In the region of the capillary, similar responses were obtained only from the limited small area of its origin. It was this area which acted in a sphincter-like manner, and frequently responded independently of the supplying arteriole or the precapillary. Such sphincter-like areas sometimes produced spontaneous rhythmic contractions as well. As no central reflex could be involved, and no ganglionic cells could be found in the membrane, the nature of this rhythmic and independent response remained undetermined.

The observations of Fulton and Lutz threw a flood of light on the subject matter of our discussion. They fell short, however, in elucidating precisely the exact nature of the mechanism of nervous control, and also in determining the bearing, if any, of the observed capillary phenomena on the circulatory mechanism of the animal.

We, therefore, made an attempt to study the nature of this nervous mechanism, and for the purpose of this study, carried on certain preliminary investigations, designed to reveal the nature of influence, if any, exerted by autonomic nerves on the mechanism of control of frog's systemic circulation.

## EXPERIMENTAL

The experiments for this purpose comprised perfusing frog's blood vessels with solutions of adrenaline chloride and of acetyl choline in different dilutions and noting the results thereof. The details of the experimental procedure adopted were as follows:—

In a weighed frog, brain and medulla-pithed, the heart was exposed and the trunkus arteriosus cleaned upto its bifurcation; a small glass canula was then inserted, away from the heart, through a small knick on the trunkus just beyond the bulb, and tied in place. It was connected by means of a rubber tube with a bulbed recepticle of known capacity, having a side-tube at its lower part. The recepticle was fixed at a height of about 6 inches above the arterial canula, and was connected at its upper end, by means of a rubber tube, with a reservoir of frog perfusion saline.

placed at a definite height above the receptacle. The perfusion was carried on by running the fluid from the reservoir through the receptacle and the arterial canula, and before the canula was inserted into the trunkus, the whole passage was filled with the fluid, care being taken to drive out all air from inside. The perfusion pressure was determined by the height of the receptacle above the arterial canula and remained unaltered throughout the experiment. The perfusion rate was controlled by an adjustable clip on the rubber tube connecting the reservoir with the receptacle, and was adjusted at 1 cc. per minute at the start. The outflow was provided through an opening in the sinus. The perfusion fluid thus entered the circulation through the opening in the trunkus, and after circulating through the body, flowed out through the opening in the sinus. The animal remained hanging vertically during the actual perfusion, being fixed to a frog-board which was clamped and secured on a vertical stand, so that a perfusion height of about 6 inches was constantly maintained during the course of the perfusion. The outflow was measured by collecting the fluid in a measuring cylinder while dripping from the toes tied together, accumulation in the tissues and the lymph sacs being prevented by incisions on the thighs and the legs, the collecting pockets and the sacs. Tissue-dehydration, during the course of the perfusion, was prevented by protecting the animal from external draught.

The effect of perfusion of the drugs, adrenaline chloride and acetyl choline, on the circulation of the animal was determined by observing the rate of outflow at varying periods, following injection of known amounts of the drugs into the receptacle through a rubber tube, clipped at its distal end and connected with the side-tube of the receptacle by the proximal end. Admixture of known amounts of the perfusion fluid in the receptacle determined the dilution in which the drugs entered the circulation of the animal.

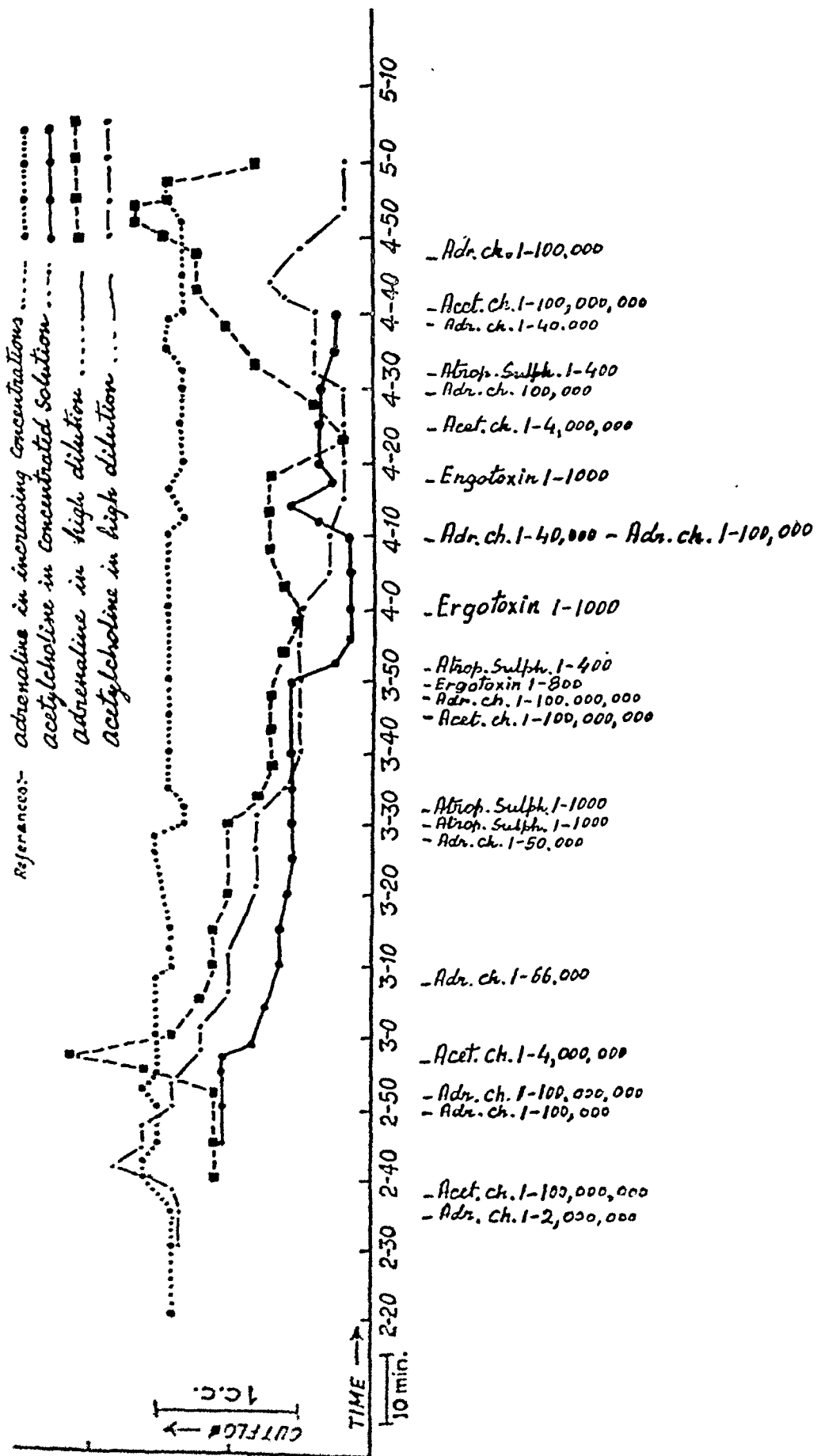
constriction ; and like adrenaline chloride, again, concentrated solutions produce immediate constriction. Atropine, as in the case of adrenaline chloride, antagonises the dilator effect, but the constrictor effect, unlike adrenaline chloride, is antagonised by atropine again, and not by ergotoxine. Thus, both the constrictor and the dilator effects of acetyl choline are antagonised by atropine ; ergotoxine antagonises neither.

### DISCUSSION

From the experimental results, as stated above, it follows that the systemic blood vessels of the frog respond to stimuli of autonomic drugs both by dilatation and constriction, the threshold for the dilator response being lower than that for the constrictor. When the concentration of such a drug goes beyond a specified limit in the circulation, the dilator response gives place to the constrictor. The blood vessels persist in their response to autonomic stimuli even in the brain and medullapithed animal ; hence, their autonomic response is independent of central control. Since these blood vessels respond by dilatation to high dilutions of both adrenaline chloride and acetyl choline, and since this dilator response to both the drugs is antagonised by atropine, it may be presumed that (1) the vessels receive a supply of cholinergic autonomic nerve fibers and that (2) in high dilutions, adrenaline chloride and acetyl choline, both stimulate these cholinergic fibers, thereby producing dilatation of the vessels. Concentrated solutions of both adrenaline chloride and acetyl choline produce constriction of the vessels. Ergotoxine appears to antagonise adrenaline constriction only, and has no influence on acetyl choline constriction. It seems, therefore, that (3) the blood vessels do receive autonomic nerve supply of adrenergic character as well ; and that (4) concentrated solutions of adrenaline chloride stimulate these adrenergic fibers in order to produce constriction of the blood vessels. The mechanism for the constrictor effect of concentrated acetyl choline solution is not well understood on the basis of the present findings ; it is quite possible that this effect also should be found to be due to adrenergic stimulation in other concentrations of the drug, and until further elaborate examinations should prove otherwise, such a possibility could not be altogether dispelled. In fact, that atropine should appear to antagonise this constrictor effect raises issues which call for further elaborate consideration.

On the basis of the present observations, however, it seems possible to suggest that (i) an autonomic drug is adrenergic or cholinergic according as its concentration in the circulation, for the time being, is high or low ; that (ii) all autonomic nerves are essentially of the same physiological nature, the character of their response as adrenergic or cholinergic being determined by the stimulus arising from high or low concentration of the causative agent at that time in the circulating blood.

Graphic Representation of the Effects of Diffusion of Frogs Blood-Vessels with  
ADRENALINE and ACETYLCHOLIN  
in High and Low Dilution



## SUMMARY

1. The mechanism which regulates the flow of blood through the systemic blood vessels of the frog has not yet been definitely ascertained, though its importance in applied pharmacology has long been recognised.

2. In order, therefore, to study the precise nature of the mechanism of nervous control, frog's blood vessels were perfused with varying concentrations of adrenalin chloride and of acetylcholin, as being authentic representatives respectively of the adrenergic and the cholinergic group of autonomic drugs.

3. The observed facts seem to suggest that (i) an autonomic drug is adrenergic or cholinergic according as its concentration, for the time being, in the circulation is high or low ; and (ii) all autonomic nerves are essentially of the same physiological nature, the character of their response as adrenergic or cholinergic being determined by the stimulus arising from high or low concentration of the causative agent in the circulation at the particular time.

4. Should these propositions be substantiated by the results of further elaborate examinations, they would introduce a new conception about the basic physiology of autonomic nerves in the amphibion, whereby a common mechanism is suggested for all autonomic responses, their character varying with the varying nature of the causative stimulus.

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## HIPPURIC ACID SYNTHESIS AS A LIVER FUNCTION TEST

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### Contents:

1. The title 'Hippuric Acid Synthesis as (a) Liver Function Test'. The aims, object and the scope of work
2. The test proper is described.
  - A. (1) Oral method
  - (2) Intravenous method
  - B. Historical aspect
  - C. Physiological considerations
3. The record of work done by the oral and intravenous methods amongst the following groups:—
  - (1) Oral method—Normal—31 subjects
  - (2) Intravenous method—(a) Normal—19 subjects
  - (b) Diseased—30 subjects.Comments on normal and abnormal findings.
4. Discussion on the work so far published on various aspects. The work is separated out in two subdivisions:
  - (1) Foreign
  - (2) Indian
5. Conclusions.
6. Summary.
7. References cited in the text.

In the following pages, the author proposes to give an account of the results of investigations carried out by him in connection with the use of hippuric acid synthesis as a hepatic efficiency test. This work has been spread over two years. It comprises of 105 recorded observations with this test with special reference to the normal values by the oral and the intravenous methods and the application of the intravenous method in certain diseased conditions.

The author was attracted to this test when it was published in the 'Recent Advances in Medicine'—Edition 1944. The test struck him as a simple one to perform and on going over the published literature, a very widely accepted one by physicians, surgeons, and obstetricians alike. It was, therefore, planned to carry out this test in all cases referred to this laboratory for the estimation of efficiency of the liver. Before undertaking the use of this test in diseased conditions as a routine, a series of tests were carried out to obtain normal values as obtainable in the local population. The normal values with the oral method was first studied in a series of 31 healthy individuals. Later, the intravenous method was carried out in a series of 19 normals. After the preliminary evaluation of the data thus compiled, the intravenous test was performed on 30 cases at this hospital. The results of this work are discussed in this paper.

The liver has a large number of physiological functions to perform. The present test relates to the assessment of its function of detoxication only. The principle underlying the basis of this test is a process of detoxication occurring in the liver. The liver detoxicates most of the endogenous and exogenous poisons circulating in the body. The synthesis of hippuric acid is one of such mechanisms, which exists in the liver. Liver is intimately concerned with the protein metabolism and forms amino-acids for the synthesis of cell proteins of the tissues. One of such amino-acids synthesised in the liver is glycine. In this test glycine is conjugated in the liver with benzoic acid to form hippuric acid. The benzoic acid is thus detoxicated and the hepatic function of detoxication is thereby assessed with this test.

## 2. The Test.

A. (1) Oral method: Quick's method (1) as described in 1932 was an oral test, performed as follows:

- (1) After emptying the bladder, give 5.9 grammes of Sodii Benzoas C.P. by mouth one hour after tea, coffee and toast in the morning. Half a glass of water is allowed soon after, if asked for.
- (2) Collect four one-hourly samples of urine.
- (3) Estimate the hippuric acid content of each specimen and of the total bulk. The specimens are to be preserved in ice-chest, if the estimation is not done immediately. Use of Toluene as a preservative is allowed.

*Estimation.*—There are two methods described by Quick in his papers on this test. The simpler one is known as the clinical method and the elaborate one is too complicated for routine use and inexperienced hands. In his experience, both the methods showed complete agreement and except for checking up his own results, he did not either use it or recommend it for routine use.

*Clinical method.*—In the simpler clinical method, the specimen is measured correct to a c.c. and is acidified with strong hydrochloric acid till the red congo red paper turns blue. Nearly 1 c.c. of acid is required. A precipitate of hippuric acid rendered insoluble at this pH is thrown out as the specimen is well stirred. It

is then allowed to stand at room temperature for one hour to allow complete precipitation. The precipitate is then filtered out on a weighed filter paper. The container is completely washed free of the precipitate by repeated washings of filtered urine. The filter paper with the funnel is then allowed to dry in air for 24 to 48 hours in a dust-proof cupboard. When dry, the filter paper is weighed with the deposit to the nearest third decimal place on the same chemical balance. On deduction of the original weight of the filter paper, the amount of insolublized hippuric acid is known. To obtain the total hippuric acid content, the amount of hippuric acid remaining soluble in the urine after acidification is now to be calculated. Quick stated that every 100 c.c. of urine can keep 0.33 g. of hippuric acid in solution after acidification. The soluble hippuric acid is, therefore, obtained by multiplying the number of c.cs. of urine by 0.0033. The insolublized and the soluble amounts of hippuric acid are then added up to give the total hippuric acid content of the urine. The results are now to be converted in terms of total benzoic acid excreted as hippuric acid. One gramme of hippuric acid is synthesized from 0.68 grammes of benzoic acid and therefore, by multiplying the grammes of hippuric acid excreted by 0.68, one gets the amount of benzoic acid excreted as hippuric acid. The results are expressed in these terms. This was the original method as described by Quick. Later work has modified this method so as to make it more accurate. In 1940, Quick has modified this method and recommends that along with the acidification of urine, 100 grammes of ammonium sulphate should be added to every 100 c.c. of urine. Under these conditions, maximum precipitate of hippuric acid is thrown out and the soluble fraction becomes constant. With this method which has been used in the investigations recorded in this paper, only 0.1 g. of hippuric acid is to be calculated as the soluble fraction for every 100 cc. of urine acidified. The factor 0.0033 thus is now replaced by the factor 0.001. Rest of the calculations as described in the original method of Quick remains unchanged.

*Elaborate method.*—The more complex method may be briefly described. Measured quantity varying from 5 to 10 c.c. of urine is taken in an extraction tube. One c.c. of 5 Normal sulphuric acid and two drops of 10% sodium tungstate are added and the extraction is done for 90 minutes with ether. Later, the ether is distilled over and few c.cs. of 20% hydrochloric acid is added to the residue. The resulting solution is refluxed on a hot plate for one hour and transferred to a small dish. It is then evaporated to dryness. To this residue, 20 c.c. of hot distilled water is added. A very small amount of activated charcoal Norit is also added. The solution is filtered. This is repeated over and again a few times with the same 20 c.c. of hot distilled water and a little of Norit, finally making up the volume of the filtrate to 20 c.c. Now one drop of 1% neutral red is added and the filtrate is neutralised to pH 7.0 i.e., till it is slightly pink. Then 6 drops of 1% phenolphthalein and 10 c.c. of neutral 40% formalin are added. The solution is titrated with 0.1 Normal sodium hydroxide till a deep red colour matches the standard prepared by mixing 20 c.c. of distilled water, 10 c.c. of neutral 40% formalin, 6 drops of 1% phenolphthalein, 1 drop of 1% neutral red and 0.3 c.c. of 0.1 Normal sodium hydroxide. Since 1 c.c. of 0.1 Normal sodium hydroxide is equal to 1 cc. of 1 Normal glycine, the titration value corrected for 0.3 cc. blank can be converted directly either to hippuric acid or to benzoic acid

While evaluating comparatively, Quick mentions that weighing to the second decimal place and titration with 0.2 Normal sodium hydroxide or Formol titration have for practical purposes in clinical pathology, no discernible difference and the simpler clinical method is the method of choice.

According to his observations, the hippuric acid excretion reaches 1 g. or more during second and third hours and the total excretion for four hours for normal American population is from 2.99 g. to 3.55 g. with an average of 3.2 g.

(2) Intravenous method: In the years following Quick's publication in 1932, much work was done in connection with this test. A detailed resume of all the work is deferred to a later part of this paper. Quick (1) published his intravenous method for this test in 1938 by a preliminary note and followed it up with later publication. The technique for the intravenous test is as follows:—

- (1) Empty the bladder
- (2) Inject the patient intravenously with 1.77 g. of sodium benzoate dissolved in 20 cc. of pyrogen-free double distilled water, taking five minutes to inject.
- (3) Exactly one hour after the start of the injection, empty the bladder.
- (4) Estimate the hippuric acid content of this specimen by the simple clinical method.

A normal American excretes 0.7 g. to 0.95 g. on an average in this test.

*Choice of the method:* Whenever a second test is developed after the first, the natural conclusion should be that the oral method had some disadvantages, of which the author of the test was conscious. Actually speaking the oral test has not much of a disadvantage and as late as 1944, much work has been published with the oral test. The so-called disadvantages are:—

- (1) The four hour period is too exhausting for the patient.
- (2) Sodi benzoas is a nauseating drug and the drug is invariably vomitted out repeatedly by those who are sensitive to gastric stimuli.
- (3) The test is less sensitive because of the factors such as absorption from the gut etc., having a vitiating influence.

The last *viz.*, the accuracy is perhaps the only solid reason by itself that speaks in favour of an intravenous method. Mateer and others (2) have published a paper in 1943, where they have shown that 85% more cases of liver damage were diagnosed by the intravenous method as compared to the oral method. This establishes the superiority of the venous route, which is now being preferred by the clinicians. So far as the absorption of sodi benzoas from the gut is concerned it has been asserted in the later work that it is absorbed fairly evenly and quickly, so much so that the delayed absorption does not occur. No vitiation of the test can, therefore, occur on this account. The venous route, therefore, scores over the oral route in speed, accuracy and comfort. During the venous technique, the patient at the most feels dizziness, flushing of the face, a hot feeling or pain along the injected vein for a moment but such minimal feeling is only occasionally met with.

*Reactions.*—There is only one authentic report on the reaction following the use of sodi benzoas for the test. Bryan (3) stated that a normal man can take as much as 25 grammes of sodi benzoas in a day without any ill effect. The very principle of this test is of detoxication. Benzoic acid is absent from our food. Body cannot metabolise the benzene ring. Yet a compound of quinnic acid occurring in prunes has the same detoxication mechanism by conjugation with glycine. The prunes have never been incriminated for any adverse reactions. As a matter of fact, benzoic acid is a stimulant for the synthesis of amino-acid glycine by the liver. The greater the quantity of benzoic acid administered as sodi benzoas, the greater should be the glycine production in the liver till a maximum is reached as is aimed in this test. Production of glycine is inevitable for the body and this mechanism starts functioning in the body ever since birth. Milk practically contains no glycine at all and hence the tissues begin the synthesis of glycine for their growth from the birth. It is, therefore, difficult to account for any such reaction as was recorded by Kingsley.

In the case recorded by Kingsley, the patient was in the hospital for haemolytic anaemia with icterus. Oral test was performed twice. Twice the reaction occurred with the same constant symptomatology and course. The stuff was used previously and later for other patients, who showed no reaction at all. Nearly an hour following the ingestion of the drug, the patient complained a vice like pain in the chest of excruciating nature. He became unconscious, developed fast pulse, sweating, fall of blood pressure, slowed respirations and cold extremities. This was more or less a symptomatology of shock. He had to be treated very energetically for three days with peripheral circulatory stimulants, plasma transfusions and oxygen therapy before he could become as he was. On recovery, the haematology showed appreciable decrease of the leucocytic count, increase of icteric index and an unaltered chemistry in blood, urine, etc. To exclude the fact that the reaction was not a coincidence only, as the literature had not described such a single case the oral test was repeated and the patient had a prototype reaction again. The improvement which he had made stopped and on recovery he went into agranulocytic condition. Autopsy did not reveal anything in particular. Kingsley summarises the reaction as being of an anaphylactoid nature. It would also appear the agranulocytic attack might have something to do with the benzene nucleus of the compound.

B. Historical aspect: Synthesis of hippuric acid has been one of the interesting problems of study for physiological chemist since the early days. Glycine and benzoic acid are the primary compounds essential for the synthesis of hippuric acid. This was known long since but most of the early research workers have devoted their energy on the probable site of synthesis, reason for synthesis and factors governing the synthesis. It was only in 1932 that Quick (1) suggested this mechanism to be used as a hepatic efficiency test and the test, therefore, is known after his name.

In 1877, Bunge and Schmiedelberg perfused the renal artery of a dog with glycine and benzoic acid and they found hippuric acid in the renal vein. This led to a belief that synthesis of hippuric acid was a function of the kidney. A late

as 1921, Kingsley and Swanson proposed hippuric acid synthesis as a test for renal function. It is quite evident that hippuric acid being a nitrogenous product, its excretion would become defective in renal damage. In 1925, Bryan (3) studied the synthesis of hippuric acid as a test for renal function and made some important observations, which did not attract his own attention but which proved to be of great help to the later workers. In his figures, he quoted reduced synthesis in a limited number of cases showing the liver damage. Prior to this and as early as 1911, Friedman had already demonstrated that the synthesis of hippuric acid could take place in the liver of the rabbits. Linking of this knowledge to Bryan's findings was fairly easy and Quick started working on this problem. In 1926 he demonstrated that hippuric acid is synthesized only in the Kidney in case of dogs but so far as the other animals and man are concerned, most of this synthesis took place in the liver by the agency of the enzymes hippuricase. He showed that very negligible synthesis also occurred in other tissues including the kidney. He further differentiated between hydrolysis of hippuric acid and the synthesis of the same. He also pointed out that benzoic acid is also excreted as glucuronic acid monobenzoate under certain conditions.

In 1931, Quick had completed the following data:

- (1) The synthesis occurs chiefly in the liver and the amount formed in any other tissues including the kidney is so negligible as to be insufficient to compensate for lessened synthesis in the liver.
- (2) The renal excretion of synthesized hippuric acid is almost hundred per cent in all normal men and this is only affected in cases of gross renal failure. The renal excretion is, therefore, only an indication of a hepatic synthesis.
- (3) The whole liver is required for an efficient synthesis and any reduction in the synthesis is an indication of damage to even a small part of the liver.

He therefore suggested that hippuric acid synthesis could be used as a test for liver function and in 1932, he published his method and the results.

### C. Physiological considerations:

Question would arise whether liver alone can form glycine and why should a normal liver produce glycine. Various workers have shown that the capacity to form glycine is mainly confined to the liver. It forms glycine not only to supply for building up of tissue proteins but also for synthesis of glycocholic acid of bile. Glycocholic acid is formed by conjugation of glycine and cholic acid. This has been one of the established functions of the liver, leave alone the detoxication. Evidently the glycine is not specially prepared by the liver in response to administration of sodi benzoas in the test. To be quite clear, test was made for glycine and glycine is not made for the test. Synthesis of glycine by liver takes place at a certain rate in a normal liver and this could be increased till a maximum could be reached, beyond which glycine production cannot increase. The test, therefore, tests the maximum capacity of the liver unlike the others where a minimal capacity is brought out by normal readings of those tests. The test is not positive in the latter case till the disease process in the liver is far advanced. On the other

hand the reduction in the maximum capacity is immediately brought out by a reduction in the synthesis value with the present test. The reduction of synthesis value is referred to as a positive test. It now, therefore, follows that this test does not suffer just because the liver has an enormous reserve power to fulfill other functions. The test measures the reserve power already and measures its reduction also. If at all anything could be offered as a criticism for this test, it is the too great a delicateness which would give a positive test arising from extraneous influences on the liver. The test is only a measure for the detoxication function of the liver and of the entirety of the liver.

Glycine is essential for body growth and is derived both from food and from synthesis in the liver. Connective tissue, gelatine and keratin are very rich sources in the food. As a rule, vegetable proteins and milk give a poor supply. In the basis of this test, exogenous ingested glycine is eliminated as the patients are only allowed tea, coffee and toast one hour before the test in the morning. Whatever glycine, therefore, is used up in the synthesis of hippuric acid in this test is an endogenous product.

Glycine, for whatever normal purpose it is formed in the liver, can be short circuited. Benzoic acid deviates it from its normal views in the body. In the test the glycine combines with benzoic acid to form hippuric acid instead of combining as it normally should combine with cholic acid to form glychocholic acid of bile. This deviation to form hippuric acid is to protect the body against benzoic acid which is harmful and which cannot be metabolised by the body tissues. By such a conjugation the liver detoxicates benzoic acid and converts it into hippuric acid, which is perfectly harmless and is perfectly easily excreted by the kidneys in the urine. This test, therefore, measures the detoxication function—an important and a vital function indeed.

The amount of sodi benzoas ingested is so adjusted in this test that the maximum amount of glycine that may be produced in the liver is conjugated with the benzoic acid and there is more than enough of benzoic acid than can be used by glycine in the process of conjugation in the period under test. As the test involves a time factor (speed of glycine production) in addition to the amount, the final time limit has been so adjusted that the constant maximum speed of glycine production is assessed. Further, allowance of time may increase the amount of hippuric acid but the rate of hippuric acid excretion would not rise. If the glycine is not available in the period of the test, some benzoic acid conjugates with glycuronic acid and is excreted as monobenzoate of glycuronic acid.

Test is also based on the fact that excretion of hippuric acid in the urine is always greater and more speedy than the hippuric acid synthesis by liver and other tissues. This is shown by the speed of excretion of sodium hippurate when injected intravenously. This test has a limitation that if the kidneys are damaged and if they do not excrete nitrogenous excretion products efficiently, the test will fail and is contraindicated because hippuric acid is a nitrogenous product.

Some facts about glycine synthesis have been studied. Glycine has the property of opening the lumen of the glomerular tufts. In its absence, the tufts do not



open and if glycine is perfused, the tufts open out in direct proportion to the glycine perfused. May be that protein deficiency of which glycine deficiency is the finest type, may cause renal damage in this way. Again if glycine is short circuited by benzoic acid and if body is not allowed its normal use, the amount of uric acid excretion in the urine is diminished. Following the performance of this test and after the ingestion of prunes, there is increased hippuric acid excretion and a diminished uric acid excretion in the urine. Gouty cases are, therefore, to be avoided for using this test for liver function. Failure of detoxication in the liver may be the probable genesis of gout. This lessened excretion of uric acid may be one of the results of deficiency of glycine, in absence of which the glomerular tufts do not properly open.

Pigs and rabbits can synthesize large amounts of glycine. Dogs synthesize glycine poorly. The synthesis has also been studied experimentally in various animals. No evidence of better synthesis is available in carnivora as compared with herbivora. It would be interesting to note that such an animal as camel has also not been an exception to the performance of investigations for the medical research. Slight differences in rate and amount of glycine production have been noted in different animals *e.g.* mice, rabbits, etc.

Some work has been done to find out the relation between the quantity of urine excreted and the hippuric acid present in the urine. The number of factors concerned in the urinary output are so many and uncontrollable that it could only be proved that the same patient excreted hippuric acid in a little varying quantity if the quantity of urine varied. The results are, therefore, inconclusive.

Production of glycine by the body is proportional to the surface area of the body. Taller and bulkier individuals can form more glycine than the shorter and thinner ones. This evidently does not vitiate the test because the ratio of the weight of liver to the body weight is unaltered in any case and hypersynthesis of hippuric acid by the bulky is no objection to the efficacy of the test. Hypersynthesis is definitely rare and a reference will be made to it in the later part of this paper. There is no correlation of synthesis to the body weight as has been observed by Machella.

### 3. Record of work.

#### (1) Oral method:

There is always a scope for variation of normal values of the different population in all biochemical standards and it was, therefore, necessary to estimate the normal values of the local population. This work was undertaken during the vacation and the volunteers available were too few. However, 36 volunteers could be enlisted. Quick's oral technique and the modified clinical method of estimation were used but in order to offer facility to the candidates volunteering, the technique used by Rennie (4) of collecting only one four-hourly sample was used. Hourly samples were collected in four volunteers only. All other directions described in the first part of this paper were strictly adhered to except for the fact that one glass

of water was allowed instead of half a glass. This was because of two considerations—hotter days of hotter climate and the habits of the people under test.

The persons taken up for the test were members of the staff of this department and medical students. The series, therefore, does not correspond to the method of taking random samples in the true statistical sense. All the same this was the only thing that could be done. The volunteers were all apparently in perfect health. A cursory physical examination was always done. None gave history of previous liver disease, jaundice, treatment with metallic drugs, addiction to liquor, a venereal disease, and drug addiction. Their Kahn's test was negative. Liver was not palpable. There was no tenderness or fullness in the right hypochondrium. None had a history suggestive of renal disease before and there was no albuminuria in any.

Four hour period was not any way trying for the healthy persons but five volunteers vomitted the drug out of 36. They had to be rejected from this series. Most of the volunteers used cardamom, pan and betel-nut to avoid nausea. There was marked variation in the amount of urine brought out at the end and some volunteers brought out excessively large amounts of urine showing the diuretic effect of the drug. The minimum four-hourly urine was 137.5 cc. The maximum was 553 cc. The average works up to 220 cc.

Below are given the values as obtained in this series of 31 healthy volunteers. The Values are expressed in terms of benzoic acid excreted as hippuric acid:

TABLE I

Number.	Values.	Number.	Values.
1	2.37	17	3.12
2	2.86	18	2.96
3	2.63	19	3.17
4	2.90	20	2.89
5	2.90	21	3.15
6	2.93	22	2.68
7	*1.96	23	3.24
8	3.43	24	3.21
9	2.88	25	2.97
10	2.97	26	3.44
11	2.95	27	3.46
12	2.96	28	3.08
13	2.68	29	2.88
14	2.69	30	2.94
15	2.97	31	2.58
16	2.99	—	—

Average: 2.62

\*If No. 7 is excluded for the reasons mentioned below the average is 2.69 or roughly 2.70

Mean: 2.97 (American 3.10, Rennie)

Standard deviation: 0.23

Co-efficient of variation: 0.77

\*No. 7 had to be excluded. It is exceptionally low. The person had a cholera like diarrhoea on the next day evening after the test. He was therefore incubating some infective disorder or had a toxic metabolic effect on the liver during the test. The synthesis was therefore low. The author tried to induce him after recovery to get the test repeated but as he had a suspicion that the drug was a cause of his severe diarrhoea, he did not get it done again.

It would have been ideal if all the tests could have been repeated on the same individuals but it was not possible. Only in five cases could the tests be repeated and these are included in table No. 1 on their average result. Results were as under:

Four one-hourly samples were collected in four cases only. The results obtained are as under:

TABLE II

Serial No.	1st.	2nd.	3rd.	4th.	5th.	Average.
4	2.87	2.76	2.97	2.88	2.92	2.90
16	3.46	3.00	2.88	2.92	2.70	2.99
17	3.21	2.90	3.31	3.06	3.12	3.12
21	2.92	3.44	3.55	2.89	2.96	3.15
28	2.74	3.24	2.98	3.34	3.11	3.08

TABLE III

Serial No.	1	2	3	4	Total.
24	0.75	0.80	1.02	0.64	3.21
25	0.78	0.67	1.08	0.44	2.97
29	0.68	0.67	0.78	0.75	2.88
31	0.63	0.78	0.72	0.45	2.58

It is not possible to draw any far reaching conclusions in such a simple work as this. Certainly the oft repeated remark that 3 g. is not the normal minimal limit, also holds good for Indians. Results expressed above are quite consistent with workers in this field abroad. Though statistically these figures do not represent a random sample, arbitrarily with the condition obtaining in this laboratory, the author is inclined to set a minimal normal limit as 2.7 g. and would consider it as hundred per cent efficiency. A variation of 90% to 120% is allowed as normal. If the test is low, it must be repeated as has been the dictum. One single low reading in absence of clinical manifestations should not be taken as indicative of hepatic damage unless the result is repeated and is consistent.

(2) The intravenous method was also followed: A normal series of 21 volunteers selected on the same criteria as in the oral method were enlisted. Quick's technique described in the first part of this paper was strictly followed. The ampoules and the solution were prepared with personal care. The injection was given by the author (except himself) and the time was carefully controlled. No catheterization has been done. Oral route is no longer a method of choice by the clinicians as the intravenous route gives some additional advantages mentioned in the previous part of this paper. It was, therefore, also necessary to study the normal values with the venous test after technique of estimations was gone through with the oral method. Members of the staff and the medical students formed the volunteers for this test. The results obtained are as under:

TABLE IV.

Number.	Values.	Number.	Values.
1	0.68	12	0.88
2	0.74	13	0.81
3	0.80	14	0.84
4	0.62	15	0.87
5	0.79	16	0.66
6	0.77	17	0.88
7	0.71	18	0.63
8	0.86	19	0.81
9	0.70	20	0.82
10	0.76	21	0.72
11	0.67	—	—

Average: 0.78

Mean: 0.75

Standard deviation: 0.04

Co-efficient of variation: 0.04

Average quantity of urine in this series was 68 cc. Here again, no far reaching conclusions can be drawn. The results are consistent but a large series with intravenous test has not been published so far in India. American value is 0.7 g. to 0.9 g. representing 90% to 120%. The author considers 0.78 g. as 100% for the tests in his laboratory.

*Values in disease.*—Thirty tests have been done on cases of various varieties referred to this laboratory. This was done not with a view to discover something new but some cases were sent up by the clinicians for hepatic efficiency test and some groups were studied with the co-operation of the clinician to see if the author could corroborate the work already done on this subject as discussed in the later part of this paper.

The results obtained were as under:

TABLE V

Diagnosis.	Total No. of Cases.	Values.
Tabercular peritonitis	1	0.77
Toxic hepatitis ( <i>arsenical</i> )	1	0.32
Infective hepatitis	6	later 0.35 0.14, later 0.41, 0.32, 0.31, 0.24, 0.14, and 0.74
Amyloid degeneration of liver	1	0.35
Atrophic cirrhosis-post-hepatitic	1	0.13
Portal cirrhosis	5	0.17, 0.14, 0.35, 0.22, 0.15
Normal pregnancy	3	0.70, 0.24, 0.24
Congestive cardiac failure	2	0.35, 0.39
Obstructive jaundice	1	0.33
Long undiagnosed jaundice	2	0.08, 0.32
Amœbic hepatitis	2	0.31, later 0.55.
Infantile cirrhosis	1	0.22
Colloid goitre	1	0.14
Subacute yellow atrophy	1	0.25
Hyperemesis gravidarum	1	0.38
Dyspepsia-neurosis	1	0.18
		0.71

The author has no remarks to offer on these results. The clinico-pathological correlation has been done in all these cases. All of these have been clinically seen by him also. The diagnosis has been entered after the discharge of the case by a final review of facts. Amongst the various factors considered, the following were considered very carefully:—

- (1) Nature of suspected disease of liver and likely pathological changes in it.
- (2) Next probable diagnosis.
- (3) History and physical findings.
- (4) Present condition of the patient with special reference to co-existent illness and septic foci.
- (5) History of jaundice—when—course of illness and progress till date.
- (6) Condition of nutrition—daily menu—whether sufficient protective foods per week per head.
- (7) Any addictions—alcohol, country liquor, toddy, opium, etc.
- (8) Family history and venereal disease.
- (9) Any previous treatment with metallic drugs or a suggestive history—occupation.
- (10) Investigations done with special reference to plasma proteins, Van den Bergh's test, Icteric Index, W. R, Prothrombin time and Liver biopsy.
- (11) Response to therapy and progress.

The author likes to point out three interesting cases, where the test helped to revise the diagnosis.

- Case No. 1 A lady, aged 27 years was admitted for ascitis, oedema of feet and breathlessness for a month. She was diagnosed as a case of cirrhosis of liver. There was hypoproteinaemia also. Intravenous test gave a synthesis value of 0.77 g. The liver biopsy did not reveal fibrotic changes but suggested focal necrosis. The diagnosis was revised as tubercular peritonitis. The patient had an enlarged lymph node in the neck. The biopsy from which showed tubercular lymphadenitis. The ascitic fluid also gave a consistent picture. But for normal synthesis, the case would have been diagnosed and treated for cirrhosis of liver.
- Case No. 2 A male, aged 35 was admitted with dyspeptic symptoms and complained of morning nausea and gastritis. The case was diagnosed as early cirrhosis. The intravenous test gave a normal value of 0.71 g. The case was finally diagnosed as one of anxiety neurosis.
- Case No. 3 An under trial male prisoner, aged 18 years, developed jaundice during arseno therapy. The case was diagnosed as subacute nodular atrophy of the liver in view of signs. The patient had a single test done before discharge. A value of 0.74 g. was obtained with the venous test. The recovery of synthesis was uneventful and the diagnosis at discharge was changed by the clinician to infective hepatitis—cured.

The general impression prevails that this test is mainly of value for diagnosis between hepatic and extra hepatic jaundice. However, this is not so and the test has much wider clinical applications as seen above. A wider usage and a follow up in certain disease groups may throw more light on obscure etiological factors of diseases like cirrhosis of various types. During these investigations hypoproteinaemias existed with a normal synthesis value. Liver biopsy failed to reveal structural changes in clinically well defined cases, while the synthesis was invariably markedly defective in them. The biochemical changes in the liver may be precursors to what is seen in the liver during life and autopsy by a histological study. Synthesis improved as the clinical condition improved and came to normal after all the signs had disappeared for some time. Prothrombin time was increased in all jaundiced cases. The arsenical hepatitis gave a positive Van den Bergh's test.

#### 4. Discussion on published data:

##### (1) Foreign work on this test:

In 1932, the test was developed by Quick, working in connection with surgical research. In his first publications, Quick performed the test in a small number of 36 cases with the oral technique. His normal value was 2.90 g. to 3.55 g. in four hours and the excretion reached 1 g. in second and third hours. He called this normal efficiency as 85% to 110%. Taking 3.2 g. as the average reading of his series, as 100%, the normal efficiency works up as 90% to 110% but being probably aware of the technical difficulties that then existed, Quick seems to have allowed 5% margin of error on safer side. He showed that a jaundice of obstructive type

could be differentiated from hepato-toxic type by this test. The hippuric acid synthesis was reduced in all cases of toxic or infective jaundice, while jaundice of cholelithiasis and cholecystitis did not reduce the synthesis in early stage. He showed that clinical disappearance of jaundice in the infective toxic group was not a true cure as the synthesis and hence the detoxication function of the liver was still deficient for some time later. Only when the synthesis came to normal, the patient is perfectly normal and free from the disease. He recorded diminished synthesis in syphilitic cirrhosis of liver and carcinoma of the head of pancreas. This was the only liver function test that showed liver damage in cases of secondary metastasis in the liver. He showed that the jaundice from whatever cause will reduce synthesis if it is sufficiently prolonged but in a jaundice of acute onset, he considered low synthesis at early stage as diagnostic of catarrhal jaundice which we now call infective hepatitis. His further work also showed that persons with 50% reduction in hippuric acid synthesis gave poor surgical risks. In his experimental studies he showed that anaesthetised dogs showed early diminished synthesis and no histological change in the liver cells while his control animals developed jaundice and histological changes at a later stage. He advances this evidence as significant of this test being a very sensitive one for liver function. All these observations stood the test of subsequent observations.

In his observations, Quick stated that portal cirrhosis of the liver always gave a normal synthesis value. Rennie on the contrary later showed that all cases of portal cirrhosis showed a diminished synthesis. In 1936 Quick also corrected his views and obtained the findings similar to Rennie. In 1936, Quick recorded nearly normal synthesis in cases of congestive cardiac failure in cardio-vascular diseases. Yardumian and Rosenthal while studying the cardio-vascular diseases showed that so long as congestive cardiac failure has not set in, the synthesis was normal but as soon as congestive cardiac failure sets in, synthesis is definitely diminished. Later, Vaccaro (5) also recorded similar findings.

Diminished synthesis is a contraindication for treatment with hepatotoxic drugs like Neosalvarsan, Mapharside, Cincophen, etc. Riddel (6) states that diminished synthesis appears much earlier than the onset of jaundice during arsenotherapy and if proper testing facilities are available this test can be easily used as a warning signal.

Hischheimer (7) studied the synthesis in cases of pregnant women in a large series. A large number of pregnant women exhibited significant reduction in hippuric acid synthesis. The test returned to normal in them after the delivery. He stressed that the test loses all its value in these cases unless a satisfactory and complete excretion of hippuric acid occurs in the liver after the intravenous injection of sodium hippurate. This check test for renal function is necessary as renal damage is more common in pregnant women than in the population. He even states that it may not even be correct to interpret this test as showing liver function in pregnant women because of the physiological changes and the needs occurring in this state. The disordered physiology of pregnancy may render it even null and void. In his series, more than half of the cases of women with uncomplicated pregnancy showed subnormal synthesis. Women with evidences of toxæmia of

pregnancy showed a still higher incidence of diminished synthesis. Toxaemic group also showed diminished power of renal excretion for hippuric acid after the intravenous injection of sodium hippurate.

The test has been studied in cases of *myasthenia gravis* and progressive muscular atrophy. It is normal in both these conditions. Evidently, therefore, glycine is not formed in the muscle and treatment by glycine for muscular dystrophy is not likely to be useful.

Yardumian (8) recorded normal synthesis in hyperthyroidism, unassociated with heart failure but Bartels states that the synthesis is always reduced in hyperthyroidism. The probable reason is the glycogen depletion due to excessive glycogenolysis arising out of the increased metabolic activity in this condition. Haines (9) also studied this problem. He noticed one additional alteration in physiology of hyperthyroidism. Not only is there a diminished synthesis but there is also disturbance in the mechanism of synthesis by the enzyme hippuricase. Whenever synthesis is reduced in other diseases such as infective hepatitis, the synthesis returns to normal if the test is done soon after the oral administration of glycine in doses of 3 g. or a feed of gelatine, which is rich in glycine. It is not possible to bring the reduced synthesis to normal in cases of hyperthyroidism to normal by any such procedure. This means that even if glycine is made available, liver cannot conjugate glycine with benzoic acid. The enzyme system may be absent or inactivated. The synthesis is reduced irrespective of the fact whether the jaundice or cardiac failure are present or not. Further, Haines has shown that it is very unwise to build an opinion on the surgical risks involved on the results of this test only. The question whether the operation should be done in one or more stages also did not alone depend on the results of this test, as in his opinion this test is so delicate that even transient functional changes in the liver upset the synthesis considerably without increasing the surgical risks.

Hippuric acid synthesis has been studied in relation to schizophrenia. Strom-Olsen (10) states that catatonia of this disease is not associated in any way with reduced synthesis. Quastel (11) in 1940, denied that truth of the above statement

Quick included 2 cases of pernicious anaemia in his original 32 tests on diseased. He recorded diminished synthesis in one and this disease was therefore studied by other workers. Fouts (12) showed that there is reduction of synthesis in this disease. He also showed that the lower value of synthesis varied irrespectively and had no influence on the dose requirements of liver extract to bring about a remission in these cases.

Most of the above work has been done with the oral test. Very few papers have been published with the venous test, which came in vogue in 1940. Much advance has been done in the technique of the test. Following Quick, failure to obtain results similar to him was commonly noted and several short comings in the performance of the test were studied and removed.

Rennie collected four hourly samples only and not one hourly for four hours. His range of normal in a very large series was 2.40 to 4.30 g. with a mean of



3.19 g. The standard deviation was 1.07 g. and the standard error  $\pm 0.13$  g. He showed for the first time that a single normal reading excludes liver damage but a single low synthesis value does not mean liver damage. The test must be repeated in cases of low values in apparently healthy persons and if the results are consistently low, there is liver damage. As low as 0.71 g. value was obtained for four hourly collections in many normal cases and these individuals had a previous or subsequent normal results, when the tests were repeated in his series. He attributes it to the failure to empty the bladder completely and recommend that catheterization should be done in all females and males with weak abdominal muscles, or enlarged prostate and in the aged.

Delor (13) who studied a number of normal values finds that 3.0 g. value of hippuric acid synthesis for four hourly collection is a minimal normal length. He considers bromsulphthalein test was a little more sensitive than the hippuric acid test. Machella (14) also endorses this view but Mateer (2) finds bromsulphthalein to be less sensitive than the hippuric acid synthesis test.

It was discovered that soluble fraction of hippuric acid after the urine is acidified is variable because solubility of hippuric acid in the urine is not constant. Quick's method of estimating this fraction for calculation by multiplying the number of ccs. of urine by 0.0033 g. was based on assumption that the solubility is constant and is 0.33 g. for 100 cc. of urine. When it was found that this sometimes meant too less, the reason for low figures obtained in many normal urines by others was understood. In 1940, Quick modified his method and recommended that along with the acidification of urine, 100 g. of ammonium sulphate should be added to every 100 cc. of urine. Under these conditions, maximum precipitate of hippuric acid is thrown out and the soluble fraction becomes constant. With this method only 0.1 g. of hippuric acid is to be calculated as soluble fraction for every 100 cc. of urine acidified. With this improved method he has now revised his original statement that the normal efficiency is 85% to 110%. His recent figures state that the normal efficiency of liver is 90% to 120%.

Albumin if present has to be removed by coagulation by boiling the urine and acidifying with few drops of 20% acetic acid. Bile also should preferably be removed. Values higher than 120% is probably due to the larger quantity of urine secreted does not signify any hepatic deficiency according to some workers. On the other hand, Rosenbach doubts this fact and calls the hyperexcretion of hippuric acid as a hyperexcitable stage of hepatic damage. When the quantity of urine is large it was recommended that it should be concentrated on a water bath to 50 cc. In view of improved method of estimating the soluble fraction of hippuric acid as described in the above paragraph, this step is now unnecessary with this method. Weischelbaum and Probststein state that should ammonium sulphate be not used, the soluble fraction of hippuric acid should be calculated as 0.55 g. for every 100 cc. of urine.

In 1940, Probststein and Londe obtained higher values in normals and came to the conclusion that 4 g. of sodi benzoas given orally was quite a sufficient dose in place of 5.9 g. Out of the dose given, 50% is excreted in first two hours and

80% in first four hours. They plotted a curve by taking time in hours and percentage excretion in a graph. Any deviation of normal shape of the curve of excretion indicates damage to the liver, though total four hourly collection may be normal.

They consider 50% excretion in two hours as a complete test and recommend adoption of this method so that the test period is cut down by two hours. Further they studied the test in children. In their opinion, 3 g. of sodi benzoas is a sufficient test dose for children weighing up to 60 lbs.

Paulson (15) obtained 16 low values of synthesis out of a series of 25 normals in a cancer clinic. His normals consisted of patients coming up for a disease unrelated to liver and kidney. The patients were aged and some must have toxic influences working on the physiology of the body. Some must be dehydrated to some extent or malnourished or cachectic. This group, therefore, does not represent a normal population and Paulson's criticism on normal values with this test is not in any case appealing or acceptable. The above review completes the summary of continental and American work published in the English language on this subject. The German, French, Russian, Australian and Mexican work has not been possible to go through because of language or inavailability of journals. From references available, it seems some work has also been done with this test in a tuberculosis sanatorium and in infectious diseases hospital. But for these exceptions, there exists hardly any published record of the test in the foreign journals.

(2) Indian work:

At the K. E. M. Hospital, Bombay, Raghavan (16) has carried out some work with this test and a preliminary report of this was published in the Indian Physician in 1943. Ten normal subjects were taken and eight out of ten showed synthesis above 3 g. The other two of the series showed less than 3 g. the lowest being 2.5 g. Many patients were taken up for the performance of the test and the results correspond fairly well with the work analysed above.

There is a publication of this test in the January issue of Antiseptic for the year 1942, from Miraj Medical Centre. Joseph in this paper reports his findings on 12 apparently healthy medical students. With the use of oral hippuric acid synthesis test, he obtained an average value of 3.1 g. He used the oral test only. The ranges are not stated but there must have been some values below 3 g. He also performed the test on 34 diseased individuals comprising of cases of cirrhosis of various varieties, catarrhal jaundice, amoebic hepatitis etc., with results similar to those quoted above.

#### SUMMARY

The test has proved useful to detect and estimate hepatic deficiency and to help the differential diagnosis as well as the prognostic inferences. It has contributed towards better understanding of the physiology of the liver. These criteria being satisfied, the test is now introduced for routine work in this laboratory.

In view of recorded facts and from the facts observed by the author in connection with this test, he concludes this paper with an observation that the use of hippuric acid synthesis as a liver function test is a very valuable and a very simple laboratory method in the diagnostic and prognostic armamentarium of a clinical pathologist. It assesses the detoxication function of the liver adequately. Since his writing this paper, an interesting paper by Dr. P. N. Wahi M.D., M.R.C.P. on study of liver disease has appeared in the Indian Medical Gazette Vol. LXXI no. 11 November 1946.

1. Result of 105 hippuric acid synthesis test for liver function have been recorded using:—
  - (a) Oral route—31 normals.
  - (b) Intravenous route—19 normals and 30 diseased.
2. Tests are described in detail.
3. A historical resume is presented on the work done in India and abroad.
4. Liver function and value of this test is discussed.
5. An attempt is made to declare this test as of value in assessing the liver damage.

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STUDIES ON CHOLINE-ESTERASE

PART III. SPECIFICITY OF CHOLINE-ESTERASE

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Regarding the work on the specificity of choline-esterase it may be mentioned that the credit goes mainly to Stedman *et al* (1), Kahane and Levy (2), Roepke (3), Vahlquist (4) and Glick (5). Shaw (6), however, criticised the conclusion arrived at by Stedman *et al* (1) about the specific nature of the enzyme choline-esterase. But Stedman *et al* (1) subsequently advanced evidence which sufficiently justified their view point regarding the specificity of choline-esterase. Recently, Nachmansohn and Rothinberg (7) furnished some definite proofs as to the specificity of choline-esterase in nervous system. No work has yet been done on the specificity of choline-ester hydrolysing enzyme present in cobra venom. Below are given some experimental findings about the absolute specificity of the enzyme in cobra venom. The pure enzyme used in these experiments was obtained by the procedure adopted by Chaudhuri (8).

## EXPERIMENTAL

*Other than esterase.*—With a view to find the action of choline-esterase isolated from cobra venom on simple ester if any, the following sets of experiments were prepared:

- A. 2.5 cc. of 1% Acetylcholinechloride and 2.0 cc. of phosphate buffer of pH 7.4 and 0.5 cc. of pure choline-esterase solution containing 1 unit e.g. 0.0508 mg.
- B. 2.5 cc. of Methyl butyrate emulsion (1 cc. in 9 cc. of normal saline) and 2 cc. of phosphate buffer of pH 7.4 and 0.5 cc. of pure choline-esterase solution containing 10 mg.

Control was always done by destroying the activity of choline-esterase at 70°C for 30 minutes and then mixing in the proportion used in actual experiments. The activity of each of the solutions was determined at an interval of 10 minutes, incubating temperature being 37°C. In addition, the activity in the case of B was also determined after a period of six hours. The readings obtained in the three experiments are tabulated below in terms of cc. of the alkali consumed after deducting the respective control figures.

TABLE I

*Strength of alkali = 0.02N Potassium hydroxide.*

Experiment.	Expt. 1.	Expt. 2.	Expt. 3.
A	1.4 cc.	1.4 cc.	1.4 cc.
B	0.00 cc.	0.00 cc.	0.00 cc.
B (after 6 hours)	0.00 cc.	0.00 cc.	0.00 cc.

A COMPARATIVE STUDY OF THE ACTIVITIES OF CHOLINE-ESTERASE AND OF OTHER ESTERASE IN CRUDE COBRA VENOM AS WELL AS OF THOSE IN NORMAL HORSE SERUM

For this experiment, the following sets were prepared:—

- A. 2.5 cc. of 1% acetylcholinechloride and 2 cc. of phosphate buffer of pH 7.4 and 0.5 cc. of cobra venom containing 1 mg.
- B. 2.5 cc. of 1% acetylcholinechloride and 2 cc. of phosphate buffer of pH 7.4 and 0.5 cc. of normal horse serum containing 72.15 mg. of protein per 1 c.c.
- C. 2.5 cc. of methyl butyrate (1 cc. in 9 cc. of normal saline) and 2 cc. of phosphate buffer of pH 7.4 and 0.5 cc. containing 10 mg. cobra venom.

- D. 2.5 cc. of methyl butyrate (1 cc. in 9 cc. of normal saline) and 2 cc. of phosphate buffer and 0.5 cc. of normal horse serum containing 72.15 mg. of protein per 1 cc. Controls were done by destroying the enzyme activities by heating at 70°C for 30 minutes and then mixing it in the proportions used in the respective sets of experiments.

Readings were taken as usual and are tabulated below in terms of cc. of the alkali required after deducting the respective control figures

TABLE II

*Strength of alkali = 0.02N Potassium hydroxide.*

Experiment.	Expt. 1.	Expt. 2.	Expt. 3.
A	1.40 cc.	1.40 cc.	1.40 cc.
B	0.36 cc.	0.35 cc.	0.35 cc.
C	0.24 cc.	0.24 cc.	0.25 cc.
D	1.26 cc.	1.26 cc.	1.25 cc.

*Other than lipase.*—To ascertain if the choline-esterase had any lipase activity the following experiments were done:

- A. 2.5 cc. of the emulsion and 2.0 cc. of phosphate buffer of 7.4 and 0.5 cc. containing 10 mg. of pure choline-esterase.
- B. 2.5 cc. of the emulsion and 2.0 cc. of phosphate buffer of pH 7.4 and 0.5 cc. of cobra venom containing 10 mg.

Controls were done as usual. Activities were determined of three sets of experiments after six hours and tabulated below in terms of cc. of the alkali consumed, after deducting the respective control figures.

TABLE III

*Strength of alkali = 0.02N Potassium hydroxide.*

Experiment.	Expt. 1	Expt. 2	Expt. 3
A	0.00 cc.	0.00 cc.	0.00 cc.
B	0.00 cc.	0.00 cc.	0.00 cc.

*Action of tributyrin as substrate.*—With the object of finding out the action of the enzyme on tributyrin as substrate the procedure utilised was that adopted by Rona and Michaelis (9) for the measure of the hydrolysis of tributyrin by liver-esterase and subsequently employed by Willstaetter and Memmen (10) in connection with the determination of the activity of the pancreatic lipase.

A straight stalagmometer with a water value of 81 at 20°C was employed. A saturated solution of pure tributyrin was used as substrate and buffered with M/3 phosphate buffer for maintaining the pH at 7.9. Two sets of experiments were done in the following way:

- (1) 25 cc. of saturated solution of tributyrin and 10 cc. of M/3 phosphate buffer at pH 7.9 and 5 cc. containing 10 mg. of choline-esterase in normal saline.
- (2) 25 cc. of saturated solution of tributyrin and 10 cc. of M/3 phosphate buffer of pH 7.9 and 5 cc. containing 10 mg. of crude cobra venom in normal saline.

Controls corresponding to each of the above set of experiments were also compared with the actual as usual. The readings were taken at the interval of 60 minutes which showed that a slight activity towards tributyrin was noticeable in the case of crude cobra venom while in the case of pure choline-esterase, activity towards tributyrin was definitely absent.

*Effect on Mixed Substrate.*—Further confirmation of specificity of choline-esterase was obtained by the use of mixed substrate as followed by Stedman *et al* (1) in the case of serum choline-esterase. The method adopted in carrying out this experiment was to estimate the activities of the enzyme separately on acetylcholine-chloride (A. C.C.) and methyl butyrate (M.B.) as substrate in the first instance and secondly with acetylcholinechloride plus methyl butyrate as mixed substrate at the interval of 10 minutes using one unit of pure choline-esterase i.e. 0.0508 mg. The amount of each of the substrates used was the same as that used in the former experiments. The readings are recorded at the interval of definite time as usual at 37°C and are tabulated below in terms of cc. of the alkali required after deducting the respective figures for controls.

TABLE IV

*Strength of alkali = 0.02N Potassium hydroxide.*

Substrate.	Choline-esterase (Pure)			Cobra venom (crude)		
	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 1.	Expt. 2.	Expt. 3.
A.C.C.	1.40 cc.	1.40 cc.	1.40 cc.	1.40 cc.	1.40 cc.	1.40 cc.
M. B.	0.00 cc.	0.00 cc.	0.00 cc.	0.01 cc.	0.05 cc.	0.04 cc.
A.C.C.	1.40 cc.	1.40 cc.	1.40 cc.	1.41 cc.	1.45 cc.	1.45 cc.
M. B.						

From the table IV, it is evident that choline-esterase was quite different from other esterases.

*Choline-esterase and Drug.*—The specificity of choline-esterase admixed with other enzymes was further confirmed in the same way by the selection of some

chemicals which inhibited the activity of the enzyme with acetylcholinechloride as substrate to a greater extent than that with methyl butyrate as substrate as was done by Stedman *et al* (1) in the case of serum choline-esterase. The substance chosen for this experiment was the drug physostigmine sulphate. The activities were first determined with cobra venom as well as choline-esterase isolated from it already inhibited by a definite concentration of physostigmine sulphate, using firstly acetylcholinechloride as substrate, secondly methyl butyrate and thirdly a mixed substrate composed of acetylcholinechloride and methyl butyrate the amount of the substrate in each case being the same as taken in the former experiments. The following sets of experiments were done in this way:

- A. 50 mg. of cobra venom in 5.0 cc. of normal saline and 1 mg. of physostigmine sulphate.
- B. 10 mg. of pure choline-esterase in 5.0 cc. of normal saline and 1 mg. of physostigmine sulphate.

Temperature was maintained at 8°C at the refrigerator for a period of twenty-four hours at pH 7.4. The activities were then determined at 37°C towards different substrates with 10 mg. of physostigmine-treated crude cobra venom and with 1 mg. physostigmine treated pure choline-esterase and the readings were recorded below in terms of cc. of the alkali after deducting the control figures.

TABLE V

*Strength of alkali = 0.02N Potassium hydroxide.*

Experiment.	A			B		
	A.C.C.	M.B.	A.C.C. + M.B.	A.C.C.	M.B.	A.C.C. - M.B.
1	0.00 cc.	0.10 cc.	0.10 cc.	0.00 cc.	0.00 cc.	0.00 cc.
2	0.00 cc.	0.10 cc.	0.10 cc.	0.00 cc.	0.00 cc.	0.00 cc.
3	0.00 cc.	0.10 cc.	0.10 cc.	0.00 cc.	0.00 cc.	0.00 cc.

#### SUMMARY AND DISCUSSION

The above results amply support the contention that the specificity of the enzyme choline-esterase isolated from crude cobra venom was absolute though Stedman and his collaborators (1) obtained a product from normal serum which got negligibly small activity towards methyl butyrate and tributyrin. But in this respect choline-esterase obtained from cobra venom was completely inactive towards substrates other than esters of choline. In addition the amount of the drug physostigmine sulphate necessary for the complete inhibition of this enzyme in cobra venom using acetylcholinechloride as substrates was responsible for only about 60% of inhibition towards methyl butyrate as substrate. This evidence also goes to prove the specific nature of the enzyme choline-esterase. Activities towards mixed substrates by the pure choline-esterase as well as the enzyme present in crude cobra venom showed further in the same way its specific nature.



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STUDIES ON CHOLINE-ESTERASE  
PART IV. INFLUENCE OF TEMPERATURE AND IRRADIATION  
ON THE ACTIVITY OF CHOLINE-ESTERASE

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Of the different factors influencing enzymic activity, temperature as well as irradiation plays an important role. Activities of most of the enzymes in aqueous solution are generally destroyed at a rapid rate even below 100°C. Miyake and Ito (1), however, showed that *aspergillus oryzae* amylase in solution was not completely deprived of its activity even when heated for two hours at 100°C. The rate of inactivation of enzymes by heat is sometimes found to be unimolecular as was observed by Arrhenius (2) in the case of dry renin and pepsin as well as of trypsin and emulsin according to Tammann (3). Each enzyme has, therefore, a temperature at which one half of its activity is destroyed in an hour which is called the critical inactivation temperature of that enzyme. Sometimes a rise of temperature increases the activity as was observed by Euler and Blix (4) in the case of catalase. Choline-esterase of cobra venom is very sensitive to heat. This will be evident from the following experiments.

Exposure to ultraviolet radiation inactivates an enzyme solution. Urease was destroyed when exposed to the ultra-violet rays as shown by Tauber (5). Seligsohn (6) had shown that hydrogen ion concentration had a great influence on the activity

of blood catalase when exposed to irradiation. Similar phenomenon was also noticed by Pincussen (7) with malt diastase and also by Uehara (8) with pepsin.

Some salts are found to have influence on the activity of the enzyme when irradiated. Kumanomidoh (9) found that the salivary amylase uninfluenced by various salts, was protected by them when the enzyme was exposed to ultraviolet rays. Pincussen (10) further showed that the enzyme was more easily destroyed in dilute solutions than in the concentrated one and also that the purified enzyme was less stable than the crude one when subjected to the action of ultraviolet rays. He also showed that in presence of potassium iodide the activity of malt amylase was more quickly destroyed owing perhaps to the liberation of iodine when under irradiation. But with pancreatic, salivary and *aspergillus* amylase the result was quite the reverse. Dreyer and Hanssen (11) found that the rate of inactivation, when the enzyme was exposed to irradiation was unimolecular with some enzymes, the rate being proportional to the amount of radiation absorbed.

No evidence has so far been furnished by any worker regarding the effect of temperature and irradiation on choline-esterase in cobra venom. The results of the experiments done in that direction are recorded below. The pure enzyme used in these experiments was prepared according to the procedure suggested by Chaudhuri (12).

## EXPERIMENTAL

### Influence of temperature

(a) *On the activity of pure choline-esterase*: 5 cc. solution of 0.2% pure choline-esterase (A) in normal saline were taken in each of a number of test tubes; pH of each solution was adjusted at pH 7.4. Another set of similar experiments was arranged side by side with 5 cc. of 0.5% solution of the pure enzyme (B). They were then heated at different temperatures for one hour. After this, the solutions in the different test tubes were cooled to room temperature and their activities determined from an aliquot portion. The results obtained in the two sets of experiments are recorded in the following table. The half-inactivation temperature of the pure choline-esterase was found to be 44°C by this procedure.

TABLE I  
*Strength of alkali=0.02N Potassium hydroxide.*

Temperature. °C.	Per cent. of original activity remaining.					
	A			B		
	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 1.	Expt. 2.	Expt. 3.
87	100	100	100	100	100	100
40	79	80	79	80	80	78
44	50	50	50	50	50	50
45	44.5	45	45	45	45	45
50	3.0	3.0	3.0	3.2	3.0	3.0
60	0.00	0.00	0.00	0.00	0.00	0.00

(b) *On crude venom*: Experiments similar to that described above with 5 cc. of each of a 0.5% (A) and of a 1% (B) solutions of crude cobra venom, was prepared and the results obtained are tabulated below. The critical inactivation temperature of choline-esterase in crude cobra venom was found to be at 45°C.

TABLE II

*Strength of alkali=0.02N Potassium hydroxide.*

Temperature °C.	Per cent. of original activity remaining.					
	A			B		
	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 1	Expt. 2.	Expt. 3.
37	100	100	100	100	100	100
40	84	85	84	84	85	83
44	53.5	55	55	55	55	54
45	50	50	50	50	50	50
50	17	16	17	16	16	17
60	0.00	0.00	0.00	0.00	0.00	0.00

#### EFFECT OF HEAT INACTIVATION IS IRREVERSIBLE

Pure choline-esterase in 0.5% solution (A) and also crude venom of the same concentration (B) inactivated by heating at different temperature were kept at 8°C in the refrigerator for 48 hours in a sterile condition. After this period the choline-esterase activities of the different solutions were determined and the results were tabulated below. It will be noticed that the activities of the solutions were not restored to their original value.

TABLE III

*Strength of alkali=0.02N Potassium hydroxide.*

Temperature °C.	Per cent. of original activity remaining.					
	A			B		
	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 1.	Expt. 2.	Expt. 3.
40	81	80	80	86	85	85
44	50	50	50	55	55	56
45	45	45	45	50	50	50
50	3	3	3.5	17	16	16
60	0.00	0.00	0.00	0.00	0.00	0.00

#### EFFECT OF PROTEIN ON HALF-INACTIVATION TEMPERATURE

10 ml. of 2% crude cobra venom solution were heated for one hour at 60°C at pH 7.4. 10 mg. of pure choline-esterase were taken in each of the six-test tubes to each of which 20 mg. of heated crude venom were added and the volume made up to 5 cc. in each case. The pH of the solution was adjusted at 7.4. These solu-

tions were heated at different temperatures for one hour. After this period the choline-esterase activity was determined as usual and are recorded in the table below. In this case the half-inactivation temperature of the enzyme was found to be  $44.6^{\circ}\text{C}$ .

TABLE IV

*Strength of alkali = 0.02N Potassium hydroxide.*

Temperature °C.	Per cent. of original activity remaining.		
	Expt. 1.	Expt. 2.	Expt. 3.
37	100	100	100
40	84	84.0	85
44	53	53.2	53
44.6	50	50	50
45	48	48.4	48
50	8.0	8.2	8.4
60	0.00	0.00	0.00

## EFFECT OF ULTRAVIOLET RAYS

(a) *On pure choline-esterase*: With the object of finding out the effect of ultraviolet rays on the activity of choline-esterase, 25 cc. of each of 0.2% (A) and 0.5% (B) aqueous solutions of the pure enzyme isolated from cobra venom were transferred to two flat porcelain basins and adjusted at pH 7.4. The solutions were then exposed to the ultraviolet rays from a mercury vapour lamp placed at a distance of 20 cm. The temperature was initially at the room temperature ( $30^{\circ}\text{C}$ ) but towards the end of the experiment, a rise of about  $1^{\circ}\text{C}$ . was noticeable. The activity in the two sets of experiments was determined at frequent intervals of time by taking out an aliquot portion. The results thus obtained are enumerated in table below and they revealed that the enzymic activity was almost completely destroyed within one hour in both the cases.

TABLE V

*Strength of alkali = 0.02N Potassium hydroxide.*

Time of exposure in minutes.	Per cent. of original activity remaining.					
	A			B		
	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 1.	Expt. 2.	Expt. 3.
0	100	100	100	100	100	100
10	84	85	85	84	85	85
20	70	70	71	69	70	71
30	54	56	55	55	55	56
45	36	37	37	36	37	37
60	20	19	19	19	19	19

(b) *On crude venom*: Similar experiments with 25 cc. of each of the two sets of concentrations of crude cobra venom such as 0.5% (A) and 1.0% (B) were repeated and the results obtained are recorded in the following table. The data showed that almost complete destruction of the enzymic activity was brought about within two hours.

TABLE VI

*Strength of alkali=0.02N Potassium hydroxide.*

Time of exposure in minutes.	Per cent. of original activity remaining.					
	A			B		
	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 1.	Expt. 2.	Expt. 3.
0	100	100	100	100	100	100
10	89	89	89	89	89	87
20	78	78	77	77	78	78
30	69	68	67	68	68	68
45	55	54	52	55	54	54
60	41	42	43	42	42	43
90	21	22	22	22	22	21
120	4	5	5	5	5	5

## EFFECT OF SALT

25 cc. of each of 0.2% solution of the choline-esterase (A) and also of 0.5% solution of crude cobra venom (B) were prepared in 5% solution of sodium sulphate and kept in two flat basins. They were then adjusted at pH 7.4 and then exposed to ultraviolet radiation. Choline-esterase activities of these solutions were determined at definite intervals of time and the results are tabulated below.

The results showed that in the presence of sodium sulphate only about half of the enzymic activity was destroyed within two hours.

TABLE VII

*Strength of alkali=0.02N Potassium hydroxide.*

Time of exposure in minutes.	Per cent. of original activity remaining.					
	A			B		
	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 1.	Expt. 2.	Expt. 3.
0	100	100	100	100	100	100
10	91	92	93	93	94	93
20	84	84	84	87	88	87
30	77	78	78	81	83	82
45	69	68	67	74	76	75
60	58	60	60	70	70	70
90	55	55	55	60	61	56
120	55	55	54	55	55	55

## SUMMARY AND DISCUSSION

An examination of the data recorded in the tables shows that the critical inactivation temperature of pure choline-esterase was  $44^{\circ}\text{C}$ , while that of choline-esterase in crude cobra venom was slightly higher- $45^{\circ}\text{C}$ . In presence of cobra venom proteins previously heated at  $60^{\circ}\text{C}$  for one hour the half inactivation temperature of pure choline-esterase was found to be  $44.6^{\circ}\text{C}$ . This slight discrepancy may be due to the fact that the crude venom is a complex substance consisting of different proteins some of which may protect the choline-esterase contained in crude venom from heat, resulting in the slight rise of the half-inactivation temperature. Further, different concentrations of the enzyme in the pure state as well as in the presence of denatured proteins prepared from crude venom showed nearly the same degree of inactivation.

Exposure to the ultraviolet rays of aqueous solutions of the pure enzyme (A) and of crude venom (B) produced almost the same extent of inactivation in both the cases. Another peculiar phenomenon observed with this enzyme was its stability to irradiation in presence of 5% sodium sulphate solution. It is evident that the salt got considerable protective action.

## ACKNOWLEDGEMENT

I like to express my gratitude to Dr. B. N. Ghosh, D.Sc., F.N.I. (Lond.), for his interest and advice.

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STUDIES ON CHOLINE-ESTERASE  
PART V. EFFECT OF SUBSTRATE CONCENTRATION AND  
TEMPERATURE ON THE RATE OF HYDROLYSIS

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The subject of enzyme-substrate reaction was approached from different angles by different workers. Barëndrecht (1) advanced the radiation theory of enzyme reaction ; but most of the pioneer workers in this line postulated the theory of the union of the enzyme and substrate. Some considered it to be a chemical union, while others supported the theory of the substrate absorbed on the surface of the enzyme. Recent advance in our knowledge on this subject especially due to Langmuir (2) has explained thoroughly and convincingly the theory underlying the enzyme-substrate reaction. Willstätter (3) put forward the idea that the enzyme molecule consisted of an active centre with a large colloidal carrier on which the substrate was absorbed.



Michaelis and Menten (4) while studying the initial velocities of hydrolysis of sucrose in different concentrations by invertase, deduced an equation which was supported by Brown (5) and others. Thus,  $K_m = x \left( \frac{V}{V} - 1 \right)$  where  $K_m$  is Michaelis' constant or  $\log. x = \log. K_m - \log. \left( \frac{V}{V} - 1 \right)$ . The negative logarithm of the substrate concentration is generally known as pS. The curve obtained by plotting the activity of the enzyme against the negative logarithm of the substrate concentration is called an activity—pS curve.

The activity—pS curve was drawn up by Glick (6) in the case of serum choline-esterase. This author confirmed the findings of Glick (6) with choline-esterase in cobra venom that the increase of the substrate concentration of acetylcholinechloride for the serum choline-esterase had no inhibitory effect, on the other hand, a slight gradual increase was noticed upto a certain limit of the substrate concentration. But in the case of a simple esterase the inhibition was observed by Bamann and Schmeller (7) as well as by Murray (8) in the presence of higher concentration of substrate as above 0.0005M for sheep liver esterase and above 0.01M for rabbit-liver esterase.

It is known that the velocity of enzyme reaction increases with the rise of temperature until an optimum is reached. Above that temperature the velocity gradually decreases. It is completely destroyed with the further rise of temperature. At the optimum temperature, velocity is sometimes maximum within narrow limit of time. As in the case of choline-esterase from serum, Glick (6) found that the optimum was obtained at 40°C but it had fallen off with time. Bovet and Bovet (9) performed recently some experiments on the effect of substrate concentration on the esterase activity of cobra venom, but no work has yet been done after choline-esterase was isolated from it in pure state. Below are furnished some experimental evidences towards that direction. The purified enzyme-choline-esterase used in these experiments was obtained according to the procedure done by Chaudhuri (10).

## EXPERIMENTAL

### EFFECT OF DIFFERENT SUBSTRATE CONCENTRATIONS

For the determination of the activity of hydrolysis with different concentrations of the substrate reaction mixtures containing one unit of the pure enzyme were prepared as follows:—

2.5 cc. of x% of acetylcholinechloride in normal saline + 2.0 cc. of phosphate buffer of pH 7.4 + 0.5 cc. containing one unit of pure choline-esterase in normal saline *i.e.* 0.0508 mg.

The extent of hydrolysis in these different solutions were measured 10 minutes after the start as usual.

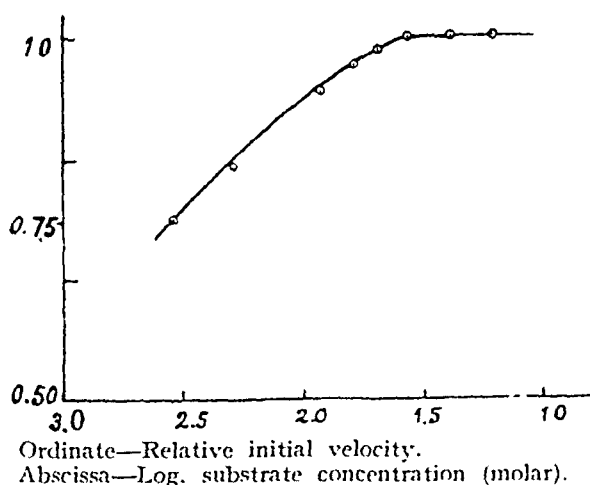
TABLE I

*Strength of alkali=0.02N Potassium hydroxide.*

Final concentration of substrate.	Choline-esterase activity in terms of cc. of 0.02N KOH Sol.			
	Expt. 1.	Expt. 2.	Expt. 3.	Mean
A. C. C.				
1.00%	1.4 cc.	1.4 cc.	1.4 cc.	1.40 cc.
0.80%	1.4 cc.	1.4 cc.	1.4 cc.	1.40 cc.
0.50%	1.4 cc.	1.4 cc.	1.4 cc.	1.40 cc.
0.40%	1.38 cc.	1.38 cc.	1.38 cc.	1.38 cc.
0.30%	1.36 cc.	1.35 cc.	1.34 cc.	1.35 cc.
0.20%	1.25 cc.	1.24 cc.	1.24 cc.	1.243 cc.
0.10%	1.15 cc.	1.15 cc.	1.16 cc.	1.155 cc.
0.05%	1.05 cc.	1.05 cc.	1.10 cc.	1.055 cc.

With crude cobra venom the same experiment was repeated. The results thus obtained fully agree with those found in the case of pure choline-esterase. Relative initial activities (mean) thus obtained from table I were plotted against the respective negative logarithm of the substrate concentrations (Molar) which represented in Fig 1 is known as activity-pS curve of the enzyme.

FIG. 1



#### EFFECT OF DIFFERENT UNITS OF PURE CHOLINE-ESTERASE

With a view to find out the rate of hydrolysis with varying units of choline-esterase in presence of a constant concentration of acetylcholinechloride as substrate four flasks containing solutions were prepared in the following way:

- (a) 2.5 cc. of 1.0% acetylcholinechloride + 2cc. of phosphate buffer of pH 7.4 + 0.5 cc. of choline-esterase solution containing 0.25 unit i.e. 0.0127 mg.

- (b) 2.5 cc. of 1.0% acetylcholinechloride + 2 cc. of phosphate buffer + 0.5 cc. of pure choline-esterase solution containing 0.5 unit *i.e.* 0.0254 mg.
- (c) 2.5 cc. of 1.0% acetylcholinechloride + 2 cc. of phosphate buffer + 0.5 cc. of pure choline-esterase solution containing 1.0 unit *i.e.* 0.0508 mg.
- (d) 2.5 cc. of 1.0% acetylcholinechloride + 2 cc. of phosphate buffer + 0.5 cc. of pure choline-esterase solution containing 2.0 units *i.e.* 0.1016 mg.

Rate of hydrolysis in each case was measured after an interval of 10 minutes from the start. The results given in the table below in terms of cc. of alkali required.

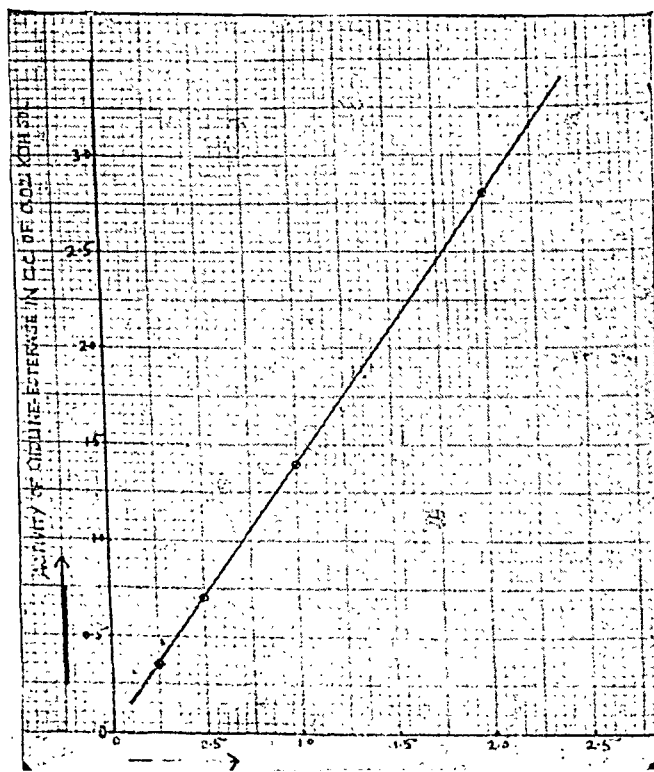
TABLE II

*Strength of alkali = 0.02N Potassium hydroxide.*

No. of the series.	Unit of pure choline-esterase.	Expt. 1.	Expt. 2.	Expt. 3.
(a)	0.25	0.35 cc.	0.35 cc.	0.35 cc.
(b)	0.5	0.70 cc.	0.70 cc.	0.70 cc.
(c)	1.0	1.40 cc.	1.40 cc.	1.40 cc.
(d)	2.0	2.80 cc.	2.80 cc.	2.80 cc.

Activities obtained in cc. of alkali in Table II are plotted against the corresponding units of pure choline-esterase which are represented in Fig. 2.

FIG. 2



## EFFECT OF TEMPERATURE ON THE VELOCITY OF REACTION

For determining the effect of temperature on the velocity of reaction catalysed by choline-esterase (pure) three sets of experiments in different flasks were arranged each containing the solutions in the proportions mentioned below:

2.5 cc. of 1.0% acetylcholinechloride in normal saline + 2.0 cc. of phosphate buffer of pH 7.4 + 0.5 cc. of choline-esterase (pure) in normal saline containing one unit (=0.0508 mg.) in each of the three flasks were kept at temperature of 37°C, 39°C and 40°C. The rate of hydrolysis was measured after definite intervals of time the results of which (mean of the three experiments in each case) after subtracting the respective control figures are enumerated below in terms of cc. of alkali required:

TABLE III

*Strength of alkali = 0.02N Potassium hydroxide.*

Time in minutes.	37°C	39°C	40°C
5	0.70 cc.	0.75 cc.	0.75 cc.
10	1.40 cc.	1.50 cc.	1.50 cc.
15	2.10 cc.	2.25 cc.	2.20 cc.
20	2.80 cc.	2.90 cc.	2.80 cc.
30	4.00 cc.	3.90 cc.	3.75 cc.
45	5.60 cc.	5.00 cc.	4.75 cc.
60	6.90 cc.	5.80 cc.	5.50 cc.

## SUMMARY AND DISCUSSION

The above results revealed the fact that the rate of hydrolysis of acetylcholine by the enzyme choline-esterase was linear for some time. But with the increase of initial substrate concentration the velocity increased slightly upto 0.5% which was different from that obtained by Glick (6) in the case of serum choline-esterase. For liver-esterase the contrary was the case as shown by many workers (7,8). Simple esterase was inhibited as the concentration of methyl butyrate was increased as shown by previous workers (7,8). From this aspect esterase was quite different from the choline-esterase.

Glick (6) found for serum choline-esterase a narrow optimum at 40°C for the rate of hydrolysis but in the case of choline-esterase purified from cobra venom the optimum was found to be at 39°C. Upto the 10th minute the velocities at 30°C and 40°C were the same but with the further increase of time the rate of reaction at 40°C decreased in comparison with that at 39°C. But the rate at 39°C. also had fallen off after the interval of 15th minute. These deviations may be judged from the standpoint of the degree of purity of the enzyme sample and the different sources from which it was obtained.

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STUDIES ON CHOLINE-ESTERASE  
PART VI. OPTIMUM  $pH$  AND STABILITY IN RELATION TO  $pH$

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Sorensen (1) in a series of papers demonstrated the influence of  $pH$  on the rate of enzymic activity. The  $pH$  at which it shows the optimum activity is dependent upon numerous factors. Nelson and Bloomfield (2) showed that an increase of temperature shifted the optimum  $pH$  of the enzyme to the more acidic region. Nature of buffer solutions used in the measurement has also a great influence. Platt and Dawson (3) found that the optimum  $pH$  for the hydrolysis of ethyl butyrate by pancreatic lipase was 6.9 in phosphate buffer, 8.5 in borate buffer. The substrate concentration may alter the optimum  $pH$  as shown in the case of honey-saccharase by Nelson and Cohn (4) and of yeast saccharase by Josephson (5). Urease from soyabean showed a change of optimum  $pH$  ranging from 7.2 to 7.9 with the change of substrate concentration of urea as determined by Lövgren (6). Willstätter and Waldschmidt-Leitz (7) observed that the addition of acid to the castor bean lipase changed the optimum  $pH$  considerably. They concluded from this that it might be due to the hydrolysis of the co-enzyme associated with the enzyme.

The inactivation of an enzyme caused by  $pH$  may be reversible in some cases. Ohlsson and Swaetichin (8) found that the activity of "taka-diastrase" was rapidly destroyed at a very low or high  $pH$ ; but when the enzyme solution was once again brought back to the  $pH$  nearer the optimum, most of the activity was restored. They put forward the view that the enzyme which might be conceived of as a colloidal protein complex dissociated at very high or low  $pH$  with the loss of activity: its original activity might be restored with the combination of the dissociated products when the excess of acid or alkali was neutralised. Different sources of the same enzyme have sometimes different optima. Phosphatase from bone and kidney has its optimum activity within the range of  $pH$  8.4 to 9.2, while the same enzyme from plant sources has the optimum  $pH$  within 3.4 to 6.0 as shown by different authors.

Plattner *et al* (9) attempted to find out the optimum  $pH$  of choline-esterase present in human serum by using pharmacological methods for the determination of its activity but were unable to establish a definite  $pH$  optimum. Bernheim and

Bernheim (10) using the same technique for its estimation found out an optimum at pH 8.4 for the choline-esterase present in the serum and brain of some of the lower animals. Glick (11) established the optimum pH at 8.4 to 8.5 for the enzyme present in human serum by an improved technique. But no worker has as yet found out the optimum pH of choline-esterase present in cobra venom. Below are given the results of the experiments done in connection with the determination of optimum pH for choline-esterase in cobra venom. The pure enzyme which was used in the following experiments was obtained by the method done by Chaudhuri (12).

### EXPERIMENTAL

#### DETERMINATION OF OPTIMUM pH USING ACETYL CHOLINE-CHLORIDE AS SUBSTRATE

With a view to determine the optimum pH of choline-esterase isolated from cobra venom, phosphate and borate buffers varying from pH 5.8 to 9.0 were used and activities determined using 0.5% phenolphthalein in alcohol from pH 5.8 to pH 7.4 and 0.5% thymolphthalein in alcohol from pH 8.0 to pH 9.0. Readings were taken at the interval of 10 minutes, the incubation temperature being 37°C. Two sets of experiments were carried out using different concentrations of acetylcholine-chloride.

- A. 2.5 cc. of 1% acetylcholinechloride in physiological saline plus 2 cc. of buffer plus 0.5 cc. of normal saline containing 1 unit of pure choline-esterase, e.g., 0.0508 mg.
- B. 2.5 cc. of 0.5% acetylcholinechloride in physiological saline plus 2 cc. of buffer plus 0.5 cc. of normal saline containing 1 unit of pure choline-esterase, e.g., 0.0508 mg.

The results obtained for the two sets are tabulated below in terms of cc. of alkali after deducting the respective control figures which were found out as usual.

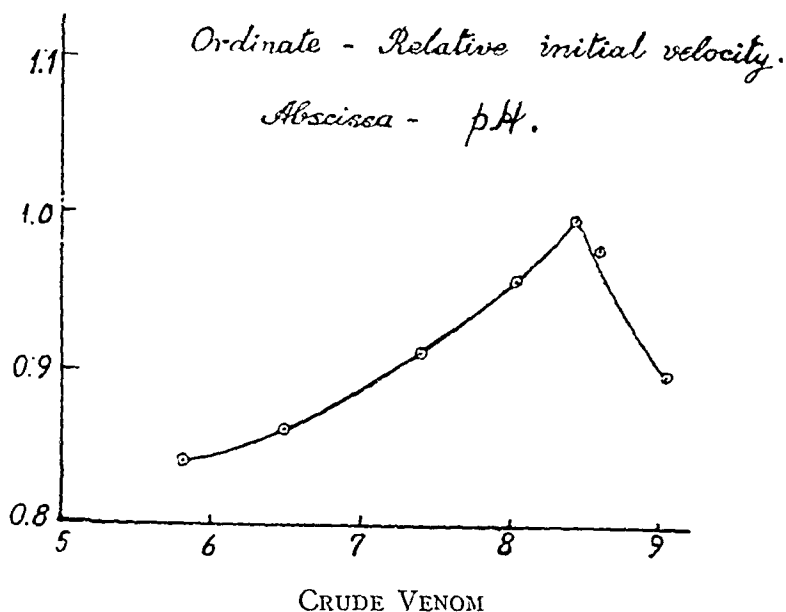
TABLE 1

*Strength of alkali = 0.02N Potassium hydroxide.*

pH	Activity of choline-esterase in terms of cc. of 0.02N KOH.					
	A			B		
	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 1.	Expt. 2.	Expt. 3.
5.8	1.30 cc.	1.31 cc.	1.30 cc.	1.22 cc.	1.24 cc.	1.22 cc.
6.5	1.34 cc.	1.32 cc.	1.34 cc.	1.26 cc.	1.25 cc.	1.26 cc.
7.4	1.40 cc.	1.40 cc.	1.40 cc.	1.30 cc.	1.30 cc.	1.30 cc.
8.0	1.48 cc.	1.48 cc.	1.46 cc.	1.40 cc.	1.40 cc.	1.40 cc.
8.4	1.54 cc.	1.55 cc.	1.54 cc.	1.46 cc.	1.46 cc.	1.45 cc.
8.5	1.50 cc.	1.50 cc.	1.50 cc.	1.42 cc.	1.44 cc.	1.42 cc.
9.0	1.35 cc.	1.36 cc.	1.36 cc.	1.30 cc.	1.30 cc.	1.30 cc.

The curve obtained by plotting the relative initial velocities (Mean) under column (A) of Table I against the corresponding  $pH$  is shown in Fig. (1) which is known as Activity- $pH$  curve.

FIG. 1



That the optimum  $pH$  was not different when crude cobra venom was used as a source of choline-esterase was shown in the results tabulated below using 0.5% final concentration of acetylcholinechloride (A.C.C.) as substrate and 1.0 mg. of crude venom under the conditions previously mentioned. Activities were determined as usual which were given in table below in terms of cc. of the alkali consumed after deducting the respective control figures.

TABLE II

*Strength of alkali = 0.02N Potassium hydroxide.*

pH	Activity of choline-esterase in terms of cc. of 0.02N KOH.		
	Expt. 1.	Expt. 2.	Expt. 3
5.8	1.30 cc.	1.30 cc.	1.30 cc.
6.5	1.35 cc.	1.34 cc.	1.34 cc.
7.4	1.40 cc.	1.40 cc.	1.40 cc.
8.0	1.46 cc.	1.46 cc.	1.48 cc.
8.4	1.55 cc.	1.55 cc.	1.54 cc.
8.5	1.52 cc.	1.50 cc.	1.50 cc.
9.0	1.35 cc.	1.36 cc.	1.36 cc.

#### CONTINUOUS TITRATION METHOD

Without using buffers and by taking recourse to the continuous titration procedure developed by Stedman *et al* (13) in the case of estimating the choline-esterase activity, the optimum  $pH$  of pure choline-esterase purified from crude cobra venom was found out which fairly agreed with that obtained by using buffer solutions.



TABLE III

*Strength of alkali = 0.02N Potassium hydroxide*

pH	Activity of choline-esterase in terms of cc. of 0.02N KOH.		
	Expt. 1	Expt. 2	Expt. 3
5.8	1.30 cc.	1.30 cc.	1.28 cc.
6.5	1.31 cc.	1.32 cc.	1.31 cc.
7.1	1.40 cc.	1.40 cc.	1.40 cc.
8.0	1.50 cc.	1.48 cc.	1.48 cc.
8.4	1.55 cc.	1.54 cc.	1.54 cc.
8.5	1.50 cc.	1.50 cc.	1.50 cc.
9.0	1.34 cc.	1.35 cc.	1.35 cc.

## STABILITY AT DIFFERENT pH

To determine the stability of choline-esterase purified from cobra venom, 0.2% solution of choline-esterase in normal saline was adjusted at different pH ranging from 1 to 13 in different test tubes by adding NaOH solution or HCL solution as the case may be. The solutions were kept at 8°C in the refrigerator in sterile condition. Activities of the solutions from different test tubes were determined as usual after definite period by first bringing an aliquot portion to pH 7.4 and then mixing it with the substrate. The results are recorded in the following table. These data were the mean of the three experiments done under the identical conditions.

TABLE IV

*Strength of alkali = 0.02N Potassium hydroxide.*

pH	Choline-esterase activity as per cent of the original.		
	1 × 24 hours.	3 × 24 hours.	7 × 24 hours.
1.0	92	83	66
3.0	96	90	81
5.0	99	96	93
6.5	100	99.5	99
7.4	100	100	99.5
8.5	98	92	85
10.0	90	72	57
13.0	81	47	20

## CRUDE COBRA VENOM

The same experiment was repeated with 0.2% crude cobra venom under the same conditions and the results obtained are given in the table below. These results recorded in the table were the mean of the three experiments done under the same conditions.

TABLE V

*Strength of alkali=0.02N Potassium hydroxide.*

pH	Choline-esterase activity as per cent. of the original.		
	1 × 24 hours.	3 × 24 hours.	7 × 24 hours
1.0	96	89	82
3.0	98	95	90
5.0	99	98	96
6.5	100	100	99.5
7.4	100	100	100
8.5	98	95	90
10.0	95	84	75
13.0	87	65	43

*pH-INHIBITED ENZYME*

The enzyme solutions at different pH after seven days incubation at 8°C were readjusted to pH 7.4 and kept at 8°C in the refrigerator for 24 hours. The activities were then determined as usual and the results thus obtained are recorded in the table below:

TABLE VI

*Strength of alkali=0.02N Potassium hydroxide.*

pH	Choline-esterase activity as per cent. of the original.					
	Crude venom.			Choline-esterase (pure)		
	Expt. 1.	Expt. 2.	Expt. 3.	Expt. 1.	Expt. 2.	Expt. 3.
1.0	100	100	99	96.0	95.0	95.0
3.0	100	100	100	99.0	98.5	98.0
5.0	100	100	100	99.0	99.0	99.0
6.5	100	100	100	99.5	99.3	99.5
7.4	100	100	100	99.5	99.5	99.5
8.5	100	100	100	99.0	98.4	98.0
10.0	100	100	100	93.0	92.0	91.0
13.0	85	81	83	63.0	61.0	60.0

## SUMMARY AND DISCUSSION

Optimum pH choline-esterase was determined using acetylcholinechloride as substrate at different concentrations in the presence as well as in the absence of buffers solutions. In all these cases the optimum pH was found to be 8.4. This result showed that buffer and substrate concentrations had no effect on the pH optimum of choline-esterase isolated from crude cobra venom.

Stability of choline-esterase in crude cobra venom and also of that purified from it was determined and it was found that the enzyme was comparatively stabler in acidic than in the alkaline solutions. Also it was noticed that the inactivating effect

of hydrogen ion was less on the impure enzyme contained in the crude venom than on the pure enzyme isolated from it. Crude venom which was a complex substance contains, besides choline-esterase, other factors which might have some protecting effect on its stability. When the enzyme solutions were brought back to pH of maximum stability viz., 7.4 and kept for 24 hours, then most of the lost activity was regained. It can be explained on the assumption as done by previous workers (8) that the enzyme protein dissociated into the simpler products at very high or low pH, resulting in the destruction of the activity temporarily; but when these dissociated products were brought back to pH 7.4, they combined to manifest almost full activity of the enzyme. Inactivation at pH 13 was not fully reversible. It may be due to the fact that apart from dissociation of the enzyme it had undergone other forms of changes such as hydrolysis or denaturation which might not be reversible.

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## ON THE ASSAY OF LIVER EXTRACTS

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Biological evaluation of whole or purified liver extracts is difficult in this country where cases of pernicious anaemia are rare. Some attempts have been made recently to study the therapeutic efficacy of liver preparations on cases of tropical macrocytic anaemia (1). But the scarcity of such cases preclude a large scale clinical evaluation, particularly with products of unknown and intermediary nature. Attempts already made by workers in the field, are still being pursued, to find out suitable method of bio-assay of liver extracts in the laboratory. This is found to be essentially necessary, for guiding research in biochemical fractionations of anti-pernicious-anaemia-factors of liver.

Several methods of assay were tried in our Laboratory in order to gain some idea regarding the efficacy of various liver preparations, stress being given to check the various processes of extraction of the anti-anaemic principles. Trials were given in rabbits, using Kruger's antigen, as well as in pigeons, rendered anaemic by administration of *B. welchii* toxin (2). But both these methods failed. Counting of reticulocytes is difficult in pigeons, owing to over lapping of various stages of reticulocytes. Ultimately, the method of Jacobson (3) using normal guinea-pig was tried. The present paper summarises an extensive series of investigations carried according to this method of assay regarding the effect of various liver extracts, on the reticulocytogenesis in guineapig. During this assay, a suitable method was required for the counting of reticulocytes with ease and accuracy.

Accordingly, a modified method was devised (4) based on that of Osgood, which gave very satisfactory results in our hands.

### EXPERIMENTAL

A total of more than a hundred guineapigs was employed in these series of investigations, the weight varying from 250-400 g. The animals were taken in groups of twelve animals in each and put on uniform diet consisting of barley, oats, wheat bran, grams, carrots and cabbage. Daily counts were done for 5-6 days, after which period, animals showing minimum fluctuation in the reticulocyte count, were sorted out. These were then grouped in sets of three, and injected with the samples of liver extracts for test, always keeping a set of animals, injected with a standard preparation of liver, as positive control. A set of animals injected with equivalent quantity of normal saline, serving as negative control was also kept for observation. After injection of liver extracts, reticulocyte counts were done daily for a period of one week.

*Standard for Comparison:* for these investigations "Neo-Hepatex", a clinically standardised liver extract was taken as the standard. This product was particularly chosen as it was found to give in our experiments, always a consistently positive response.

*Data regarding animals:* Prior to the starting of tests on liver extracts an idea regarding the average reticulocyte count of normal healthy guineapigs along with their day-to-day variation was taken on a group of thirty guineapigs, selected by random sampling. The following data were obtained:—

Average reticulocyte count of the group	= 0.64%
Probable Error (P.E.) according to Peter's formula	= 0.25
P.E. $\times$ 3.2	= 0.2

The reticulocyte percentages in individual animals of this group varied from 0.25% to 2.3%. In other groups selected for assay, it was later found that in sick animals the percentages are sometimes very high rising even upto 6-7%. The day-to-day variations in majority of animals fluctuated between 0.4 to 1.3%. In selecting animals for assay of liver extracts only those were chosen which showed the minimum fluctuation in the daily counts, the mean having a figure near about the average found for the normal population. From the statistical analysis of the figures of normal guineapigs, it was expected that under no circumstances, a carefully selected animal would show a normal variation of reticulocytosis higher than 1.44, and hence a response of 1.5% or higher of reticulocytes, after injection of test liver extract was taken as meaning a definite positive response. Though the increase in the percentage of reticulocyte after injection did not hold a linear relationship with dosage, rather it tended to be of an "all or none" type, yet it was possible to observe, that a higher dosage always gave a somewhat higher reticulocyte response than that given by the lower minimum effective dose. The purpose of dosage was, however, to find out the minimum quantity of the liver preparation which gave a positive reticulocyte response in guineapigs and comparisons were made on the basis of that quantity.

*Samples taken for test:* The following sample of liver preparations and other products were taken for assay. These were all prepared in the Laboratory.

1. Fractionated bovine liver extract (F.L.) representing 20 g. of raw liver per cc. (vacuum concentrated).
2. Fractionated liver extract (F.L.C.) representing 40 g. of raw liver per cc. (vacuum concentrated).
3. Fractionated bovine liver preparation (F.L.O) representing 20 g. of raw liver per cc. (concentrated without vacuum).
4. Proteolysed liver (P.L) representing 3 g. of raw liver per cc. (vacuum concentrated).
5. Calf liver extract, (concentrated without vacuum) representing 20 g. per cc. of raw liver.
6. Casein hydrolysate containing equivalent quantity of nitrogen per cc. as that of liver hydrolysate (P.L).
7. Physiological normal saline for negative control.

Test solutions were diluted in saline, whenever necessary, and injected intraperitoneally to find out the minimum effective dose. The volume of each injection was kept constant at 1 cc. As the nitrogen content of the liver preparations were somewhat different, the dosage was not adjusted in terms of either the nitrogen content or in terms of the raw liver that corresponded to each cc. of the extract. The dosage was graded in serial dilutions of the extracts with reference of a fixed proportion of the dilution in terms of body weight. This proportion was 0.8 cc. per kg. and was injected intraperitoneally. Each test was repeated in at least two or three animals, and concordant results in two out of the three are recorded as definite responses. With each set of experiments, the standard, "Neo-hepatex" in its highest effective dilution was always used for comparison. Tables I to V summarise the results of all these experiments.

TABLE I

*Giving the reticulocyte of response the injections of "Neo-Hepatex" (standard) or its dilution in doses of 0.8 cc. per kg. body weight.*

Percentage of reticulocyte after injection.

Dilution of test substance.	Original			1/50			1/100			1/250			1/500			1/750		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Animal.																		
Initial Mean.	0.5	0.56	0.45	0.6	0.7	0.4	0.6	0.3	0.28	0.4	0.28	0.35	0.6	0.6	0.3	0.2		
Days after injection.	1	1.2	0.8	1.0	1.1	1.3	1.2	1.3	0.8	0.9	1.2	0.6	0.7	0.9	0.8	0.7	0.5	
	2	2.2	1.0	1.8	2.5	3.0	2.5	1.8	1.1	1.5	1.8	0.9	1.0	1.2	0.6	0.5	0.3	
	3	2.8	2.5	3.0	3.2	2.9	2.1	2.3	1.9	2.1	2.3	2.0	1.2	1.5	0.7	1.0	0.3	
	4	2.5	2.4	3.4	2.7	2.5	2.0	2.0	1.7	2.0	1.9	1.5	1.3	—	1.0	0.6	0.6	
	5	2.0	2.3	1.4	1.9	2.0	2.0	1.9	1.5	1.9	1.5	0.7	1.4	1.0	—	—	—	
	6	1.7	2.0	1.1	1.0	1.7	1.5	1.4	1.2	1.5	1.3	0.5	0.9	0.9	0.7	0.6	0.4	
	7	1.8	1.9	1.0	1.5	1.7	1.3	1.2	1.4	1.2	0.8	0.5	0.4	—	—	0.5	—	

TABLE II  
*Reticulocyte response after fractionated bovine liver extract (F.L.) in same dosage, as in Table I.*  
Percentage of reticulocytes after injection.

Dilution of test substance.	1/50			1/100			1/250			1/500			
Animal No.	1	2	3	1	2	3	1	2	3	1	2	3	
Initial Mean.	0.6	0.4	0.3	0.3	0.4	0.5	0.6	0.6	0.8	0.33	0.7	0.6	
Days after injection.	1	0.8	0.9	1.2	0.5	1.0	1.2	0.8	1.3	1.1	—	—	0.7
	2	2.1	1.3	1.1	0.5	1.3	1.5	1.1	1.5	1.2	0.76	0.6	0.7
	3	1.8	1.4	2.3	1.2	1.4	2.5	1.16	1.5	1.3	0.6	0.5	0.65
	4	2.3	1.4	1.7	2.1	1.5	3.0	1.6	1.0	0.9	0.5	0.5	0.5
	5	—	1.1	—	1.5	1.2	2.1	—	1.1	0.8	—	—	—
	6	1.8	0.9	1.2	—	—	—	1.3	0.9	—	0.6	0.5	0.5
	7	1.2	—	—	1.2	1.2	1.5	1.1	—	0.8	0.58	—	—

TABLE III  
*Reticulocyte response after injection of fractionated liver extract (F.L.C.) in same dosage as in Table I.*  
Percentage of reticulocytes.

Dilution of test substance.	1/100			1/125			1/500			1/750		
Animal No.	1	2	3	1	2	3	1	2	3	1	2	3
Initial mean.	0.3	0.6	0.4	0.6	0.3	0.6	0.6	0.7	0.5	0.3	0.7	0.4
Count after injection on days.	1	0.9	1.2	0.9	—	1.0	0.9	1.0	1.0	0.5	0.6	0.6
	2	2.3	1.8	1.2	1.5	1.6	1.2	1.3	1.5	0.4	0.7	0.5
	3	1.8	1.5	1.4	2.1	2.5	1.5	1.4	2.1	0.5	0.9	0.5
	4	2.0	—	1.0	2.0	1.6	1.4	1.2	1.5	—	0.6	—
	5	1.5	1.3	1.2	—	—	1.0	—	—	0.4	—	0.4
	6	—	—	—	1.6	1.3	1.2	1.2	1.1	—	0.7	0.4
	7	1.0	0.8	0.9	1.2	—	—	—	—	0.4	—	—

TABLE IV  
*Reticulocyte response after injection of Proteolysed Liver Extract (F.L.) in same dosage as in Table I.*  
Percentage of reticulocytes.

Dilution of test substance.	1/50			1/100			1/250		
Animal No.	1	2	3	1	2	3	1	2	3
Initial Mean	0.25	0.9	0.4	0.4	0.5	0.32	0.45	0.51	
Count after injection on days.	1	6.0	1.2	1.3	1.3	1.2	0.6	0.9	0.73
	2	7.0	2.8	1.2	1.9	1.8	1.1	0.82	1.2
	3	6.0	—	—	—	—	—	—	—
	4	5.0	2.5	2.4	2.0	1.9	1.0	1.3	1.2
	5	5.5	2.2	1.8	1.9	1.7	0.8	0.8	0.9
	6	2.8	2.0	1.8	—	1.7	—	0.8	—
	7	2.0	2.0	1.5	1.3	1.2	0.7	—	0.7

TABLE V

*Average Reticulocyte response after injection (0.8 cc. per kg.) of Calf Liver Extract, Bovine Liver Extract (F.L.O), Casein hydrolysate, and physiological saline, in groups of 3 animals.*

Dilution of test substance.	Percentage of reticulocytes.					
	Calf Liver Extract.		F.L.O.		Casein hydrolysate.	Physiological saline
	0	1/25	1/2	1/50	0	0
Initial reticulocyte count.	0.6	0.54	0.48	0.5	0.61	0.56
1	0.9	0.8	1.3	0.7	0.62	0.71
2	1.3	0.93	1.8	0.9	0.59	0.80
3	1.8	1.0	2.5	1.1	0.34	0.48
4	2.3	0.8	2.1	1.1	—	—
5	2.0	0.9	1.8	0.8	0.54	0.52
6	1.5	0.64	1.4	0.9	—	—
7	1.2	0.6	1.4	—	0.38	0.6

## DISCUSSION

The purpose of this study was mainly an attempt at confirmation of the guinea-pig method of assay of liver preparation as evolved by Jacobson (*loc. cit.*). The procedure was followed on the same lines as his with certain modifications to suit the environmental conditions. In a general way, it can be taken from the tables that evaluation of the active principles of liver, can certainly be done by this method. More particularly, the method serves to differentiate different strengths of the preparation, as well as the different modes of preparation. Thus it can be seen that while vacuum concentration of the fractionated liver extracts (F.L. and F.L.C) exert definite reticulocytogenic response, the same preparation, prepared by open concentration (F.L.O) brings down the activity to a low figure. The same low activity is found with the product, calf liver extract, which exerts reticulocyte response only when injected at the original strength. It can also be noted that neither casein hydrolysate which contain the same amount of nitrogen as the proteolysed liver preparation nor the physiological saline does produce any effect on the normal variation of reticulocytes in guineapigs.

A critical analysis of the results, however, shows wider variation in the responses of reticulocytes to injection of liver extract than that reported by Jacobson. It can be seen that the animals required per kilogramme body weight quantity of extract equivalent to at least 20 mg. of raw liver, whereas Jacobson has noted positive response even in dose of 0.06 mg/kg. Taken in equivalent amounts in terms of raw liver, proteolysed liver appears likely to exert a better reticulocytogenic action than the fractionated liver extract (F.L) or even the standard, "Neo-Hepatex". Another significant difference in the response noted in our animals, is that repeated injections of liver extracts diminish rather than increase the sensitivity of the animals. Therefore, it is preferable to test samples on a large number of animals. The final



inference that can be drawn from these studies, however, is that although this method of assay gives a fairly correct idea regarding the efficacy of various principles of liver or their therapeutic possibility during fractionation, it can only serve as a qualitative method or at best a relative quantitative one. This is apparent from the results of fractionated liver extracts such as F.L. and F.L.C. The ultimate value of liver should certainly be tested on clinical cases, either of pernicious anæmia or the tropical macrocytic-anæmias.

### SUMMARY

1. An attempt has been made for the biological evaluation of various liver extracts based on the reticulocyte response of normal guineapigs, according to the technique of Jacobson.
2. The method serves as an useful qualitative method, or at best a relative quantitative one.
3. Proteolysed liver extract appears to give a better reticulocyte response in guineapigs than that exerted by fractionated and purified liver extracts.

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**PRELIMINARY OBSERVATIONS ON CERTAIN BIOCHEMICAL  
FINDINGS IN CIRRHOSIS OF THE LIVER**

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Rich and Hamilton (1) discussed the possibility of cirrhosis of liver being the result of long continued deprivation of essential dietary factors. Blumberg and McCollum (2) demonstrated the protective effect of choline against liver cirrhosis in rats. The effects of choline deficient diets and their prevention and cure in animals

have been thoroughly studied in 1942. The various biological functions of choline *i.e.*, lipotropic effect, growth promotion etc., involve several mechanisms. Lowry *et al* (3), produced liver cirrhosis in rats by a diet with low choline and casein in diet. Fatty cirrhotic livers in dogs fed a low protein diet supplemented with Vitamin B Complex could be improved by supplying large quantities of choline. The combination of large amounts of choline and liver extract rapidly overcame these signs except the fibrosis of liver.

These investigations lead us to the question of the place of choline in human nutrition, how far its deficiency could be judged in man, and whether choline deficiency is likely to be the cause of liver cirrhosis in man. Some data were provided regarding the inter-relationship of serum lipides and choline chloride administered. No definite information is available regarding the relationship of choline deficiency and the severity of the disease. The levels of lipides and much less of lipide phosphorus were studied by very few workers in biliary cirrhosis, infectious hepatitis and extensive degeneration of liver.

Our present knowledge on the subject is sufficiently advanced so far as animals are concerned and we have every reason to believe that the levels of lipide fractions and plasma proteins are closely related to this disease. For a number of years choline has been known to be a constituent of the phospholipids, lecithin and sphingomyelin but its importance in lipide metabolism as an essential dietary factor have been recognised only recently. The present investigation was undertaken with a view to finding (1) the levels of various constituents in the blood of patients suffering from liver cirrhosis and (2) whether lipide phosphorus in blood can be used as an index of the choline content in blood which may later be developed into a liver efficiency test.

## EXPERIMENTAL

The biochemical investigations were done on well established cases of portal cirrhosis of liver. No selection was made of early cases. Cases of doubtful nature were excluded. The diagnosis was made on clinical grounds and all cases had well marked ascites.

In the history one interesting feature was noted. Four of the cases complained of severe night blindness. The percentage of this complaint appears to be remarkably high when compared with other patients suffering from chronic diseases in the same hospital. Five of the patients gave a history of deep jaundice (without pain) 2 to 10 years previous to admission. The duration of jaundice was measured in weeks or months and never in days. The possibility of infective hepatitis having occurred in them cannot, therefore, be excluded.

Apart from the biochemical investigation tabled, other routine investigations were carried out. As they revealed nothing significant, they are not mentioned. All cases had low blood pressure.

Blood drawn from the vein of the patient selected, was oxalated and the analyses of various constituents were carried out on the same day. Total proteins, albumin, globulin and fibrinogen were carried out by the method of Nesslerisation and occasionally checked by the Kjeldahl method. Cholesterol was estimated by the modified method of Myers using the colorimetric estimation of Liebermann-Burchard (4). The lipide phosphorus was done by the classical method of King (5). The results are given below.

The following are the biochemical deviations observed in the present investigation.

1. Total proteins are slightly diminished.
2. Reversal of albumin-globulin ratio.
3. No significant change in cholesterol (total) and fatty acid.
4. Lipide phosphorus is diminished.
5.  $\frac{\text{Lipide P}}{\text{Cholesterol}}$  and  $\frac{\text{Lipide P}}{\text{Fatty acid}}$  are diminished.

#### DISCUSSION

The results confirm the observations made by several workers regarding the reversed albumin-globulin ratio. The levels of cholesterol do not show any regularity. Possibly the determination of free and esterified cholesterol is of greater significance since the transport of fat is indicated by the esterified cholesterol. What is most striking however, is that the level of lipide phosphorus is definitely low; in most cases less than 50% of the normal level of 10-11 mg/100 cc.

This low level of lipide phosphorus can be ascribed to a low level of choline in blood in view of the very recent and extensive investigations of Taurog, Chaikoff and Entenmann (6). For a number of years it has been known that choline is a constituent of the phospholipides lecithin and spingomyelin. Since phospholipides are particularly important in the transport of fatty acids from the liver to the tissues, it is likely that the lipotropic effect of choline is due to its capacity to form phospholipides. This is supported by the experiments of Perlman and Chaikoff using radio active phosphorus. Choline phospholipides are, therefore, intimately connected with the transport of fatty acids.

If choline deficiency is one of the factors in the production of cirrhosis of liver, the determination of the level of choline in blood would be extremely useful. As

TABLE I

TABLE I												
No.	Name	Total % Proteins	Albumin %	Globulin	Fibrogen	Cholesterol	Lipide Phosphorus	Lipide as fatty acid	Albumin Globulin	Lipide P		Remarks
										Cholesterol	Fatty acid	
Normal												
		6.5 to 8.2	4.6 to 6.7	1.2 to 2.3	.3 to .6	150-190	12 to 14	290-420	about 3	.08	.04	
1.	M.P.	4.1%	.78%	3.53%	.13%	400 mg.	5.8 mg.	310 mg.	.22	.014	.019	
2.	B.D.	5.5	2.3	2.99	.15	400	4.8	280	.79	.012	.017	
3.	M.N.	4.7	1.5	3.09	.16	156	5.4	185	.49	.035	.029	
4.	P.B.	8.8	1.3	7.34	.13	175	4.2	340	.19	.024	.012	
5.	H.M.	6.4	2.4	3.90	.16	220	5.0	355	.62	.023	.015	
6.	R.R.	6.4	2.0	4.10	.25	205	4.8	290	.50	.023	.017	
7.	A.S.	4.6	1.4	2.8	.4	205	5.0	330	.53	.024	.015	
8.	R.D.	5.2	1.6	3.26	.3	100	4.8	320	.51	.048	.015	
9	R.R.	5.7	2.0	3.2	.5	140	6.4	350	.62	.046	.018	
10.	G.R.	4.8	1.2	3.1	.5	150	5.1	300	.39	.034	.017	
11.	I.D.	6.1	2.2	3.7	.2	200	5.2	290	.59	.026	.018	
12.	B.G.	5.0	1.7	3.6	.65	120	5.2	310	.49	.043	.017	
13.	G.S.	6.8	2.0	4.3	.41	200	4.4	290	.49	.022	.015	
14.	M.K.	6.4	1.9	4.2	.33	220	4.8	285	.46	.022	.017	
15.	V.R.	6.2	3.8	2.1	.31	190	4.1	320	1.75	.022	.013	
16.	J.N.	5.4	2.0	3.0	.44	180	7.0	300	.67	.039	.023	
17.	M.K.	5.5	2.1	3.0	.4	140	5.0	310	.70	.036	.016	
18.	H.M.	5.2	1.8	3.2	.2	145	5.1	340	.56	.035	.015	
19.	S.D.	5.0	1.4	3.4	.2	335	4.8	290	.41	.014	.016	
20.	R.S.	5.8	2.3	3.3	.2	300	5.1	310	.70	.017	.016	
Average Deviation		5.7 -0.8	1.85 -1.2	3.54	.31	209	5.1 -6.9	305	.58	.028	.017	

yet, no direct and reliable method has been found. However, the experiments of Taurog *et al* (7) suggest a very useful indirect method of determining choline in blood from lipid phosphorus values. The phospholipides of plasma are broadly classified into (a) lecithins, (b) sphingomyelins and (c) cephalins. Of these, the first two are choline containing and the last is non-choline-containing. The content of each of them has been investigated since 1930. The results reported in literature varied from 58% to 78% for the choline containing phospholipides. Doubts were however cast, about the correctness of the various methods employed by the workers.

Taurog *et al* (8) after very extensive investigations arrived at two independent methods for their estimation and their accuracy was confirmed by recovering known amounts of the substances which were previously added. Lipid phosphorus was determined by the classical method of King (same as employed in this series of experiments). The molal ratio, choline/lipid P was calculated for each sample of phospholipides in sixteen specimens. This ratio represents the fraction of phospholipides containing choline. This was found to range between .95 to .98 indicating that almost all the phospholipide of plasma are choline-containing. The non-choline-containing phospholipides amount to less than 5%. Considering the very extensive nature of the experiments of Taurog *et al* and the various checks they employed, it seems reasonable to assume that their results are much more reliable than those of the predecessors.

Since the molal ratio choline/lipid P is unity, the percentage of choline can be obtained by multiplying the level of lipid P by 139.5/31 (i.e.) Mol. weight of choline/. At weight of phosphorus. In the present series of experiments involving the plasma of patients suffering from liver cirrhosis, the level of lipid phosphorus has been found to be about 5 mg. i.e., 50% of the normal level, on the average. Assuming that the molal ratio choline/lipid P—1, the level of choline in blood of these patients would be

$$\frac{5 \times 139.5 \text{ mg./100 cc.}}{31} = 22.5 \text{ mg./100 cc.}$$

These experiments, therefore, suggest that the level of lipid P in blood may be used to assess the level of choline and may be useful as suitable liver efficiency test. Moreover it suggests that choline deficiency in diet as a probable factor in the etiology of cirrhosis of liver and its use in treatment is indicated. However extensive investigation is necessary before these tentative suggestions can be put on factual basis.

#### SUMMARY

Biochemical changes in blood in clinical cases of cirrhosis of liver at the Orissa Medical College hospital have been investigated and diminution of blood protein, reversal of albumin/globulin ratio and marked diminution in Lipid P have been found. The significance of the last change is discussed and the estimation of lipid phosphorus is suggested to determine the choline deficiency. Etiology of liver cirrhosis in human cases may be due to lack of choline in diet and choline therapy is therefore suggested as a rational treatment.

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OBSERVATIONS ON ASCORBIC ACID. PART VI.—OXALIC ACID AS AN  
EXTRACTANT OF ASCORBIC ACID FROM PLANT MATERIALS AND  
ITS EFFECT ON THE STABILITY OF ASCORBIC ACID

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The extraction and stabilization of ascorbic acid during its determination has long been a problem. Most of the information in the literature relates to the ability of various acids to stabilize ascorbic acid in pure solutions. Of the different acids recommended for the stabilization of pure ascorbic acid solutions oxalic acid (1—4), metaphosphoric acid (5—7) and a mixture of metaphosphoric acid and trichloroacetic acid (8) were most satisfactory. But very little attention seems to have been devoted to the extraction of ascorbic acid from plant materials and its subsequent stabilization. The experiments described here were undertaken in order to study extractive and stabilizing ability of oxalic acid in comparison with hitherto used acid mixture (metaphoric and trichloroacetic acid) in plant materials. The technique used for the determination of ascorbic acid was according to method described earlier (Ahmad, Qureshi and Babbar, 9). The results of the investigation are given in the following tables.



TABLE I

*Loss of ascorbic acid from plant extracts in presence of oxalic acid 0.4% and acid mixture.*

Plant Material	Extracting acid	Ascorbic acid mg./100 g.		
		Initial	After 48 hrs.	Loss %
1. Peas ( <i>Pisum sativum</i> )	Oxalic acid	25.0	14.5	42.0
	Acid mixture	37.0	28.5	34.0
2. Potato ( <i>Solanum tuberosum</i> )	Oxalic acid	24.4	22.0	9.02
	Acid mixture	17.8	12.7	34.2
3. Fenugreek ( <i>Trigonella fœnugræcum</i> )	Oxalic acid	200.0	200.0	0.0
	Acid mixture	200.0	200.0	0.0
4. Aunra	Oxalic acid	26.6	8.0	69.9
	Acid mixture	32.0	26.6	16.6
5. Lemon ( <i>Citrus limonum</i> )	Oxalic acid	80.0	64.0	20.0
	Acid mixture	53.3	45.7	12.3
6. Radish leaves ( <i>Raphanus sativus</i> )	Oxalic acid	140.0	130.0	7.14
	Acid mixture	175.0	160.0	8.57
7. Bengal gram ( <i>Cicer arietinum</i> )	Oxalic acid	110.7	94.1	15.0
	Acid mixture	160.0	100.0	37.5
8. Bitter gourd ( <i>Momordica charantia</i> )	Oxalic acid	183.3	110.7	39.6
	Acid mixture	183.3	110.7	39.6
9. Rape seed leaves ( <i>Brassica napus</i> )	Oxalic acid	100.0	88.8	11.2
	Acid mixture	101.4	88.8	12.42
10. Indian Gooseberry ( <i>Phyllanthus emblica</i> )	Oxalic acid	53.3	50.0	6.2
	Acid mixture	53.3	50.0	6.2
11. Red turnip leaves ( <i>Brassica</i> )	Oxalic acid	133.3	117.0	12.22
	Acid mixture	133.3	100.0	25.00

TABLE II

*Loss of ascorbic acid from plant extracts in presence of oxalic acid (0.4%) and acid mixture in different lengths of time (3 to 5 hours).*

Plant material	Extracting acid	Ascorbic acid mg./100 g. fresh plant material.			Loss of ascorbic acid %	
		Initial.	After 3 hrs.	After 5 hrs.	After 3 hrs.	After 5 hrs.
1. Guava ( <i>Pisidium guajava</i> )	Oxalic acid	200.0	200.0	197.53	0.0	2.47
	Acid mixture	205.8	205.8	205.8	0.0	0.0
2. Lemon ( <i>Citrus limonum</i> )	Oxalic acid	41.2	41.2	40.0	0.0	2.91
	Acid mixture	52.6	52.6	51.2	0.0	1.66
3. Aunra	Oxalic acid	22.5	22.5	20.0	0.0	9.09
	Acid mixture	24.1	24.1	22.9	0.0	4.97
4. Potato ( <i>Solanum tuberosum</i> )	Oxalic acid	20.0	20.0	18.8	0.0	6.0
	Acid mixture	19.7	19.7	18.3	0.0	7.1

TABLE III

*Loss of ascorbic acid from plant extracts in presence of oxalic acid (0.4%) and acid mixture with added copper (.0002%) after 24 hours.*

Plant Material	Extracting acid	Ascorbic acid mg/100 g. fresh plant material		Loss of ascorbic acid %
		Initial	After 24 hrs.	
1. Lemon ( <i>Citrus limonum</i> )	Oxalic acid	80.0	64.0	20.0
	Oxalic acid + Cu	—	53.3	33.3
	Acid mixture	53.0	45.7	12.3
	Acid mixture + Cu	—	14.6	72.4
2. Mossammi	Oxalic acid	20.0	13.3	33.3
	Oxalic acid + Cu	—	10.0	50.0
	Acid mixture	22.8	18.5	18.8
	Acid mixture + Cu	—	15.6	31.14
3. Potato ( <i>Solanum tuberosum</i> )	Oxalic acid	24.4	22.2	9.02
	Oxalic acid + Cu	—	17.7	27.4
	Acid mixture	32.0	12.7	34.2
	Acid mixture + Cu	—	7.4	58.4
4. Dried peas ( <i>Pisum sativum</i> )	Oxalic acid	7.57	7.03	7.1
	Oxalic acid + Cu	—	6.15	18.8
	Acid mixture	8.9	7.81	12.02
	Acid mixture + Cu	—	5.79	23.7
5. Pear ( <i>Pyrus communis</i> )	Oxalic acid	5.6	4.5	19.6
	Oxalic acid + Cu	—	4.0	28.5
	Acid mixture	4.8	3.9	18.7
	Acid mixture + Cu	—	3.2	83.3
5. Orange ( <i>Citrus sinensis</i> )	Oxalic acid	53.3	26.65*	50.0*
	Oxalic acid + Cu	—	15.33*	75.5*
	Acid mixture	47.2	42.4	10.17
	Acid mixture + Cu	—	35.1	25.65
7. Lintel	Oxalic acid	12.5	10.0	20.0
	Oxalic acid + Cu	—	8.33	25.4
	Acid mixture	14.92	11.2	24.7
	Acid mixture + Cu	—	10.5	20.5
8. Palak leaves	Oxalic acid	13.3	11.4	14.2
	Oxalic acid + Cu	—	8.0	39.8
	Acid mixture	20.7	16.1	22.2
	Acid mixture + Cu	—	14.5	31.78
9. Guava ( <i>Pisidium guajava</i> )	Oxalic acid	200.0	187.8	6.1
	Oxalic acid + Cu	—	170.0	15.0
	Acid mixture	205.8	196.4	4.51
	Acid mixture + Cu	—	184.7	10.03
10. Chowli leaves	Oxalic acid	26.0	8.0*	69.2*
	Oxalic acid + Cu	—	4.0*	85.0*
	Acid mixture	23.0	—	—
	Acid mixture + Cu	—	—	—
11. Aunra	Oxalic acid	26.6	8.0*	69.2*
	Oxalic acid + Cu	—	8.1	70.1
	Acid mixture	32.0	26.6	17.2
	Acid mixture + Cu	—	16.0	40.0

\* After 48 hours.

Table I and II give the results of the extractive and anti-oxidant capacities of oxalic acid and acid mixture, consisting of metaphosphoric acid and trichloroacetic acid for varying lengths of time (24 and 3-5 hours respectively). It will be seen that in most of the plant tissues the extractive powers are comparable initially in presence of both the extracting acids. Upto 3-5 hours the final values of plant extracts are also comparable (Table II). But after 24 hours there is an appreciable difference in some plant extracts in the amounts of ascorbic acid in the presence of the two extracting acids (Table I). Generally, the values do not differ much from each other, even after 24 hours. This shows that the extractive and anti-oxidant capacities of the two acids is almost the same except in a few cases.

In Table III is given the effect of the two acids in inhibiting oxidation of ascorbic acid in presence of small concentrations of added copper. Here again the final values in some cases are comparable. In some plant extracts, the loss of ascorbic is more in the presence of oxalic acid, while in some extracts more loss is observed in presence of acid mixture. At this stage this behaviour of the two extracting acids in presence of copper cannot be satisfactorily explained.

### SUMMARY

A comparison is made of oxalic acid and a mixture of metaphosphoric acid and trichloroacetic acid with regard to their extracting and antioxidant ability of ascorbic acid in some plant extracts. It is found that oxalic acid is equally good, if not better than the most commonly used acid mixture, as the best extractant and antioxidant of ascorbic acid. Oxalic acid is also found to be equally effective in inhibiting the oxidation of ascorbic acid in plant extracts in the presence of added copper.

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## COPPER CONTENT OF SOME INDIAN FOODSTUFFS

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A number of clinical investigators have reported that some patients with hypochromic anemia do not respond to iron therapy unless copper is also given (1); others claim that with combined treatment, smaller amounts of iron are effective than when iron is given alone (2). By using double reticulocyte method, Wohl (3) has found in several cases, that copper initiated a second reticulocyte response.

The above observations prove that copper occupies a very important place in the group of trace elements which are essential for human nutrition.

The present work was undertaken to determine the copper content of some common Indian foodstuffs and in order to find out whether the requirements of copper for adults and infants are satisfied by the diets they usually consume. The copper contents of different varieties of Bengal fishes have already been determined by Saha (4) and hence fish was omitted from this work.

Samples were collected from three different sources in Calcutta and the average value for each foodstuff is expressed in this paper.

### EXPERIMENTAL

The colorimetric method of Callan and Henderson (5), using Sodium diethyl dithio-carbamate, as modified by McFarlane (6) was followed in this investigation. Ashing, extraction and development of colour were carried out according to the method described by High (7). The colour matching was carried out in an Evelyn Photoelectric Colorimeter, using Filter 420 (violet). The salient features of the method are as follows:

The dry ashing was done in a silica or platinum basin at  $600^{\circ}$ — $800^{\circ}$ C. For the extraction of the ash, a mixture of 2 volumes of dilute HCl (1+1) to 1 volume of dilute  $\text{HNO}_3$  (1+1), both of A.R. quality was used. Citric Acid was used to prevent interference by calcium phosphate and manganese. Sodium pyrophosphate was used to prevent interference by Iron. Instead of using a 2% aqueous solution of sodium diethyl-dithiocarbamate, a 1 : 25 mixture of sodium diethyl-dithiocarbamate to sodium chloride (A.R.) was prepared and 0.5 g. of this mixture was added to develop the colour. High (5) has determined that for the full development of colour containing 94 microgramme of copper, 20 mg. of sodium diethyl-dithiocarbamate is sufficient. Hence 0.5 g. of this mixture is sufficient for the estimation of copper in the range present in most foodstuffs.

In case of milk, meat and eggs, direct extraction with trichloroacetic acid, according to Tompsett (8) was done instead of acid digestion. Saha (4) found good agreement in results according to this method and that obtained after digestion with sulphuric acid and perchloric acid. The clear filtrate obtained after centrifuging was treated exactly in the same way as was done with the extract of the ash.

The results are calculated from a standard curve prepared by using different concentrations of standard solutions of cupric sulphate (A.R.). A blank figure for all the reagents was determined beforehand. Glass distilled water was used throughout the investigation.

The results of some recovery experiments are presented in Table I.

TABLE I  
Recovery of added copper from ash solution.

Copper added (microgramme)	Copper found (microgramme)	Recovery %
—	15.5	—
50	64.2	97.4
50	63.8	96.8
50	64.4	97.8
50	62.7	94.4
50	63.1	95.2

TABLE II  
Copper Content of some common Indian Foodstuffs

Common English Name	Botanical Name	Copper Microgramme/g.
<i>CEREALS</i>		
Barley (pearl)	<i>Hordeum vulgare</i>	1.3
Maize (yellow)	<i>Zea mays</i>	3.8
Millet	<i>Sorghum vulgare</i>	3.6
Rice raw milled	<i>Oryza sativa</i>	1.66
Rice parboiled milled	"	1.9
Rice parboiled hand pounded	"	1.8
Rice puffed	"	1.5
Wheat flour refined	<i>Triticum vulgare</i>	2.1
Wheat flour whole	"	3.0
Wheat whole	"	3.5
<i>PULSES</i>		
Bengal gram	<i>Cicer arietinum</i>	6.8
Green gram	<i>Phaseolus radiatus</i>	7.6
Lentil	<i>Lens esculentus</i>	5.5
Red gram	<i>Cajanus indicus</i>	7.1
Soya bean	<i>Glycine hispida</i>	3.01
<i>NUTS AND OIL SEEDS</i>		
Almond	<i>Prunus amygdalis</i>	5.1
Cocoanuts	<i>Cocos nucifera</i>	0.5
Ground nuts	<i>Arachis hypogea</i>	4.2
Mustard seeds	<i>Brassica juncea</i>	3.1
Walnut	<i>Juglas regia</i>	7.5
<i>VEGETABLES—GREEN LEAFY</i>		
Amaranth	<i>Amaranthus gangeticus</i>	6.1
Cabbage	<i>Brassica oleracea capitata</i>	1.6
Coriander	<i>Coriandrum sativum</i>	1.8
Drum stick leaves	<i>Moringe oleifera</i>	1.1
Fenugreek leaves	<i>Trigonella foenum graecum</i>	0.9
Lettuce	<i>Lactuca sativa</i>	1.8
Mint	<i>Mentha viridis</i>	1.5
Pumpkin leaves	<i>Cucurbita maxima</i>	1.4
Radish leaves	<i>Raphanus sativus</i>	1.2
Spinach	<i>Brassica cordifolia</i>	1.6



## DISCUSSION

It is evident from Table II that copper is widely present in foodstuffs. The following are good sources of copper: Pulses, nuts, cereals, leafy vegetables, flesh foods and liver. Liver is the richest source examined in the present series of foodstuffs. Milk is poor in copper, particularly human milk.

The requirement of copper as recommended by the National Research Council of U.S.A. (9), is 1 to 2 mg. daily for an adult and 0.05 mg. per Kg. of body weight for the infants and children.

It will be of interest to know the approximate content of copper in a well 'Balanced' diet for an adult. Table III shows the composition of a well 'Balanced' diet for an adult and the quantity of copper derived from each group of foodstuffs.

TABLE III  
*The contribution of copper by different groups of foodstuffs  
to a well 'Balanced' diet.*

Name of foodstuffs.	Quantity Ozs./day	Copper mg.
Rice parboiled	16	0.9
Pulses	3	0.5
Vegetables—leafy	4	0.8
Vegetables—others	4	0.2
Fruits	2	0.05
Milk	8	0.5
Fish	4	0.15
		Total—2.48 mg.

It is evident that the intake of copper is quite adequate. It may be presumed that the effect of cooking in iron vessels, as done in Bengali households, increases the copper content of foods due to contamination of copper from the vessel, as iron utensils do contain traces of copper.

As regards the diet of infants, milk is the only source of copper. The copper content of human milk is 0.016 mg. per 100 ml. An average newborn infant in India weighs 5 lb. Usually by 5th month, it doubles its weight, so that by 5th month it weighs 10 lb. According to the recommendation of the National Research Council, its requirement of copper will be 0.05 mg. of copper per Kg. body weight i.e. 0.23 mg. of copper per day. Its milk requirement will be about 2½ ozs. per pound of body weight per day (10), i.e. about 25 ozs. If infant is breastfed, it will get about 0.114 mg. of copper per day. Therefore, there is a great probability of copper deficiency, unless supplement of egg yolk, vegetables soup etc. are given.

In case an infant is fed on cows' milk, the position is perhaps not altered, because while there is a lowering of the copper content due to the dilution of milk with water, this effect is offset by the traces of copper present in metal utensils, used for boiling, finding their way into the milk.





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## SUMMARY

1. Copper content of 75 common Indian foodstuffs have been determined.
2. The possibilities of copper deficiency in adults and infants are discussed.

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## ON PLANT PHOSPHATASES. PART VI. PURIFICATION AND CHEMICAL NATURE OF LEAF PHOSPHATASE

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It has long been recognised that most plant tissues contain phosphatase having the capacity of hydrolysing phosphoric esters, but intensive study of the purification and chemical nature of the enzyme has hitherto been almost exclusively concerned with the enzymes from animal sources. The phosphatases of higher plants and particularly the leaf phosphatases have not received the attention that their ubiquity demands.

Pfankuch (1) purified the phosphatase from potato and sugar beet by fractional precipitation with alcohol and the purified preparation had an activity of 5-10 PE/mg. and 10-15 PE/mg. respectively. Later, Giri (2) obtained highly active preparations of phosphatase from sprouted soyabean (*glycine hispida*). The method of purification consisted of (a) fractional precipitation with acetone (b) solution of the most active fraction (c) removal of the inert material by precipitation at pH 5.0 and (d) final dialysis or ultra-filtration. The purified preparation possessed an activity of about 150 phy PE/mg. For glycerophosphate hydrolysis, it was about 350 times as active as the original seed power. The purified preparation gave protein and carbohydrate colour reactions.

There is practically no work reported in literature on the purification of leaf phosphatase and its chemical nature. The information available in literature regarding the phosphatases of plant leaves is frequently confined to records of its presence, and of the optimum pH for its actions. Some attention has been paid to possible differences in phosphatase activity. Since leaves in general are very potent sources of the enzyme among plant tissues, it was thought desirable that leaf phosphatase should be selected for purification. The present paper records the various steps involved in the purification of phosphatase from French bean leaves and the results of the study of its chemical nature.

## EXPERIMENTAL

(a) *Preparation of leaf material.* Fresh leaves of French bean plant (*Phaseolus vulgaris*, L) were used for the isolation and purification of the enzyme. The dry leaf powder was prepared from the leaves according to the method described by the authors (Das and Giri, 3).

(b) *Extraction of the enzyme.* The enzyme was extracted with water from the dry leaf powder as described before (Das and Giri, 3).

(c) *Phytophosphatase unit (Phy. Ph. U.).* The unit of phytophosphatase activity is defined as the amount of enzyme required for the formation of 0.1 mg. of inorganic phosphorus after one hour's hydrolysis at 35°C under optimum conditions of substrate concentration (6%) and pH 5.2.  $\beta$ -glycerophosphate was used as substrate.

The phosphatase activity is always expressed in terms of mg. of inorganic P formed in the total volume of the reaction mixture unless otherwise stated.

The phosphatase activity was determined as described before (Das and Giri, 4).

For purposes of convenience, the phosphatase activity was determined at 0.4% substrate concentration and the value thus obtained was multiplied by a factor so as to obtain the value corresponding to the activity at optimum substrate concentration (6 per cent). It was found that the activity at 6% substrate concentration was always four times the activity at 0.4% substrate concentration. The value obtained for activity at 0.4% substrate concentration was, therefore multiplied by 4 in order to obtain activity at optimum substrate concentration (*i.e.* 6 per cent).

## I. PURIFICATION OF THE ENZYME

Much preliminary work was done to determine the most promising procedure for purification of the enzyme. We need not discuss here in detail all the experi-

mental work which has entailed in the development of our method for the purification of the enzyme. Some of the important results are, however, briefly described below.

### I. *Method of preliminary purification.*

Aqueous extract of the leaf powder was used as starting material for the purification of the enzyme. Various methods of purification were studied. All the methods of purification studied namely, fractional salting out with ammonium sulphate, fractional precipitation with alcohol and acetone yield products having practically the same potency. The loss in enzyme activity during the course of purification by these methods is very negligible. The method of alcohol precipitation was, however, used in all subsequent experiments.

### II. *Purification by adsorption.*

(a) *Adsorption of the enzyme.* Various adsorbents like Fuller's earth, norite charcoal, alumina B, alumina C $\gamma$ , magnesium hydroxide, tricalcium phosphate were investigated for finding out the specific nature of the adsorbents for leaf phosphatase. It was found that Fuller's earth and norite charcoal do not adsorb the enzyme while alumina B, alumina C $\gamma$ , magnesium hydroxide and tricalcium phosphate adsorb enzyme from the aqueous solution at pH 7.0. On further investigation it was found that alumina C $\gamma$  is the most convenient and effective adsorbent for the enzyme and this was, therefore, used for the purification of the enzyme. Experiments on the influence of pH on adsorption of the phosphatase by Al<sub>2</sub>O<sub>3</sub>C $\gamma$  showed that the percentage adsorption of the enzyme increases with decrease in pH of the solution, the adsorption being complete at pH 3.6. The adsorption was therefore, carried out at pH 3.6.

(b) *Elution:* After adsorption the elution of the enzyme without loss in activity was next investigated. It was found that the enzyme could not be eluted from the adsorbent by any of the known elutrients generally used, namely, phosphate buffer (pH 7.2), CO<sub>2</sub>, sodium borate, acetic acid, acetate buffer (pH 4.4-4.8) and sodium acetate at pH 5.2-6.8 however, elutes the enzyme to a small extent. In this connection it may be of interest to refer to the work of Schmidt and Thannhauser (5) who showed that phosphatase once adsorbed on alumina C $\gamma$  cannot be recovered in soluble form. They have, therefore, used the adsorbent for removing contaminating substances leaving the enzyme in solution, the adsorbent being added in very small quantities. We have, however, succeeded in liberating the enzyme from alumina C $\gamma$  by using salts of carboxylic acids as elutrients (4).

Among the salts of carboxylic acids investigated that of tartaric acid was found to be the best, the enzyme being completely eluted from Al<sub>2</sub>O<sub>3</sub>C $\gamma$  at 10% Sodium tartrate concentration.

The method of purification finally worked out was based upon these properties.

### III. *Final Method of Purification*

#### I. *First Stage: Fractional precipitation with alcohol.*

*Fraction I.*—100 cc of fresh aqueous extract of french bean leaf powder was mixed well with 20 cc of cold re-distilled absolute alcohol for 5 minutes and the

precipitate formed was centrifuged immediately. The precipitate thus obtained is designated as *Fraction I*. The clear centrifugate was used for further fractionation. The precipitate (fraction 1) was dissolved in 100 cc of cold water and aliquots were taken for the determination of the activity and dryweight.

*Fraction II*.—The centrifugate from *Fraction I* was mixed well with 30 cc more of cold redistilled absolute alcohol for 5 minutes and the precipitate formed was centrifuged immediately. The centrifugate was discarded. The precipitate, which is designated as *Fraction II* was dissolved in 100 cc of cold water and the activity and dryweight were determined.

## 2. Second Stage : Adsorption and elution.

80 cc of the enzyme solution of *Fraction II* were taken and 40 cc of M/5 acetic acid—acetate buffer of pH 3.6 were added together with 20 cc of aluminol gel (containing 151.2 mg of  $Al_2O_3 \cdot C\gamma$ ) and 20 cc of distilled water. After frequent shaking for half an hour, it was centrifuged. The centrifugate was discarded. The residue containing the alumina-phosphatase adsorbate was treated with 10 per cent sodium tartrate solution, and shaken frequently for half an hour in order to elute the enzyme from the adsorbents. It was then centrifuged. The centrifugate which contained the enzyme was then subjected to further purification by dialysis.

## 3. Third Stage : Dialysis.

The enzyme solution after elution was then dialysed for 40 hours in collodion bag against distilled water at 5°C. After dialysis the activity and dry weight determination of the enzyme solution were carried out.

The progress of the purification is shown in Table I.

The results show that highly active preparations of the enzyme can be obtained by the methods of purification described above. Various steps involved in the purification procedure adopted can be summarised as follows:—

1. Fractional precipitation with alcohol.
2. Solution of the most active fraction.
3. Absorption of the enzyme from the aqueous solution of the most active fraction at pH 3.6 by  $Al_2O_3 \cdot C\gamma$  gel.
4. Elution of the enzyme from the  $Al_2O_3 \cdot C\gamma$  phosphates adsorbate by 10% sodium tartrate solution.
5. Dialysis.

By adopting the above procedure for the purification of the enzyme 140-160 fold increase in activity can be obtained and the final product possessed an activity of 139 phy P.U./mg. which is about 160 times as active as the original leaf powder.

## II. GENERAL PROPERTIES OF THE PURIFIED ENZYME

### 1. Influence of hydrogen ion concentration on the activity of the enzyme.

The reaction mixtures contained 10 cc acetic acid—acetate buffer (m/5) of varying pH, 10 cc substrate (1%), 2 cc of enzyme and 3 cc of water. The pH of the reaction mixtures was tested potentiometrically. The reaction mixtures were incubated at  $35 \pm 0.2^\circ C$  for one hour and the inorganic P. liberated was determined. The results are presented in Table II.

TABLE I  
*The progress of purification.*

Experiment No.	Total Phy. P.U. in 10 gram of leaf powder used for extraction with 100 cc. water.	Total Phy. P.U. in 100 cc. of the original extract.	Original Aqueous Extract.	Phy. P.U./mg.	Stages of Purification.	Quantity in mg.	Activity Phy. P.U./mg.	Yield in Phy. P.U.	Description of Fractions.	Total yield in Phy. P.U.	Phy. P.U. per mg. of dry leaf powder.
V	10120	10000	200.0	2.96	1st Stage.—						
					(a) <i>Fraction I.</i> (precipitate obtained by adding 20 cc. alcohol to 100 cc. enzyme).	194	16.0	3104			
					(b) <i>Fraction II.</i> (precipitate obtained by adding 30 cc. alcohol to the centrifugate from fraction I).	336	18.88	6343.6		9447.6	1.01
					2nd and 3rd Stage.—	43.2	142.52	6156.9			
					Enzyme solution from Fraction II was adsorbed at pH 3.6 on alumina gel and then eluted with 10.0% Na-tartrate. It was then dialysed for 40 hours in ice chamber.						



Experiment No.	Original Aqueous Extract.			Stages of Purification.		Description of Fractions.		Yield in Phy. P.U.		Total yield in Phy. P.U.	Phy. P.U. per mg. of dry leaf powder.
	Total Phy. P.U. in 100 cc. of the original extract.	Activity of the extract Phy. P.U./2 cc.	Phy. P.U./mg.	Quantity in mg.	Activity Phy. P.U./mg.	Yield in Phy. P.U.					
B	9600 Gram of leaf powder used for extraction with 100 cc. water.	9156	183.12	2.8		146 414 46	20 16 138.4	2920 6624 6366.4	9544	0.96	
				1st Stage.— (a) Fraction I. (b) Fraction II.							
				2nd and 3rd Stages.— Enzyme solution from Fraction II was adsorbed at pH 3.6 on alumina gel and then eluted with 10.0% Na-tartrate. It was then dialysed for 40 hours in ice chamber.							
C	8600	8344	166.88	2.4		108 396 44	20.8 17.2 139.2	2246.4 6811.2 6124.8	9057.6	0.86	
				1st Stage.— (a) Fraction I. (b) Fraction II.							
				2nd and 3rd Stages.— Enzyme solution from Fraction II was adsorbed at pH 3.6 on alumina gel and then eluted with 10% Na-tartrate. It was then dialysed for 40 hours in ice chamber.							
D	8000	8344	166.88	2.4		116 444 84	24.5 14.9 42.84	2842.0 6216.0 3598.6	9058	0.86	
				1st Stage.— (a) Fraction I. (b) Fraction II.							
				2nd and 3rd Stages.— Enzyme solution from Fraction II was adsorbed at pH 3.6 on alumina gel and then eluted with 10% Na-tartrate. It was then subjected to ultrafiltration.							

TABLE II

*Influence of hydrogen ion concentration on the activity of the leaf phosphatase*

pH	Phosphatase activity in mg. P.
3.6	1.3
4.0	1.54
4.21	1.80
4.6	1.95
5.2	2.00
5.7	1.91
6.2	1.7

The results show that the optimum pH of the phosphatase activity lies at 5.2. It was reported earlier that the optimum pH of the phosphatase activity of the leaf powder was 5.2. Thus the optimum pH of leaf phosphatase does not vary with the purity of the enzyme. This observation is contrary to the findings of Ignatieff and Wasteneys (6) who found that the optimum pH for phosphatase activity of the leaf of Canada Wonder beans lies at 5.5—5.9.

2. *Effect of substrate concentration on the activity of leaf phosphatase and the determination of Michaelis constant.*

Experiments on the effect of substrate concentration on the activity of leaf phosphatase (Table III) have shown that glycerophosphate hydrolysis by the enzyme, obeys strictly Michaelis and Menten's equation and the initial velocity of hydrolysis can be predicted by the theory that glycerophosphate enters into combination with the enzyme phosphatase. The constants  $V_{max}$  and  $K_s$  were evolved by the graphical method described by Lineweaver and Burk (7).

TABLE III

*Effect of substrate concentration on the activity of leaf phosphatase and the determination of Michaelis constant.*

Sodium- $\beta$ -glycerophosphate concentration (S) M	Mg. P. in 25 cc. of reaction mixture after one hour hydrolysis.	Calculated from the equation. $V = \frac{1.54 (S)}{(S)0.012}$
0.013	1.05	1.09
0.026	1.67	1.73
0.0322	2.08	2.0
0.0645	2.63	2.74
0.0968	3.05	3.16
0.13	3.43	3.43
0.16	3.58	3.59
0.20	3.75	3.75
0.226	3.85	3.83
0.26	3.81	3.9

It is clear from the above table that the values calculated according to the Michaelis and Menten's equation for the activity of the enzyme at the corresponding substrate concentration agree well with the observed values.

### 3. *Relative rate of hydrolysis of various substrates by leaf phosphatases.*

The activity of leaf phosphatase was determined using different substrates (Sodium- $\beta$ -glycerophosphate, (Merck); Sodium Alpha glycerophosphate (Boots Pure Drug Co), Sodium Hexose diphosphate, prepared from Calcium hexose diphosphate, Sodium pyrophosphate) at pH 5.2 and at temperature  $35 \pm 0.1^\circ\text{C}$ . The reaction mixtures contained 10 mg. of total phosphorus irrespective of the substrates used. The reaction mixtures contained 10 cc. of acetate buffer pH 5.2; substrate containing 10 mg. of phosphorus; enzyme and water to make up the total volume to 25 cc. After 1 hour hydrolysis the inorganic P in the reaction mixture was determined. The results are shown in Table IV.

TABLE IV

*Relative rate of hydrolysis of various substrates by the leaf phosphatase.*

Time in minutes.	Mg. of P. formed in the total volume of the reaction mixture.			
	Sodium hexose diphosphate.	Sodium alpha glycerophosphate.	Sodium- $\beta$ -glycerophosphate.	Sodium Pyrophosphate.
0	0	0	0	0
5	0.152	0.356	0.46	0.95
10	0.27	0.563	0.8	1.75
15	0.34	0.75	0.93	2.26
20	0.40	0.85	1.06	2.8
30	0.75	1.02	1.65	3.75
40	0.80	1.21	1.9	4.06
60	0.92	1.44	2.32	5.8

The results show that among the substrates, pyrophosphate is more rapidly hydrolysed than all the other substrates. The rate of hydrolysis of  $\beta$ -glycerophosphate is greater than the Alpha glycerophosphate. Hexose diphosphate is slowly hydrolysed by the enzyme. The order of increasing hydrolysability of the substrates by the leaf phosphatase is as follows:—

Pyrophosphate >  $\beta$ -glycerophosphate > Alpha glycerophosphate > Hexose diphosphate

### III. THE CHEMICAL NATURE OF THE ENZYME

The preparation used for studying the chemical nature of the enzyme was obtained by purification according to the method described above.

#### 1. *Qualitative tests for carbohydrates and proteins.*

100 cc. of the purified enzyme preparation was evaporated to 10cc. and used for the following tests.

*Molisch reaction*—positive.

*Fehling's test*—The enzyme solution does not reduce Fehling's solution. But after hydrolysis with 10% HCl for one hour, it gives positive reduction test. This indi-

cates that the enzyme solution contains polysaccharides or glucosides which liberates reducing sugars on hydrolysis with acid.

*Biuret reaction*—Negative.

*Nin hydrin reaction*—Positive.

*Nitroprusside Test for thiols*—Negative.

*Total nitrogen*—3.3 per cent.

2. *Digestion with papain*—The activity of the enzyme is not affected by digestion with papain.

3. *Influence of various substances on the activity of leaf phosphatase which are known to combine with the various groups in the enzyme molecule.*

The effect of iodine, iodoacetic acid, iodoacetamide, potassium permanganate on the hydrolysis of glycerophosphate by the enzyme was investigated. Reaction mixtures were set up containing 10 cc. of M/5 acetate buffer (pH 5.2), 5 cc. (4%) sodium- $\beta$ -glycerophosphate, 5 cc. of the enzyme and known volume of the substance whose influence is to be studied on the enzymic hydrolysis. The total volume of the reaction mixture was made up to 25 cc. The time of hydrolysis was 1 hour. The results are presented in Table V.

TABLE V

*The effect of various substances on the hydrolysis of glycerophosphate by the leaf phosphatase.*

Substances added.	Concentration of the substances added.	Phosphatase activity.		Inhibition %
		Control.	Treated.	
Iodine	2.5 mg.	2.01	0.37	82.0
	0.5 mg.	2.01	0.88	56.2
Iodoacetic acid	10 mg.	2.01	2.0	0
	25 mg.	2.01	2.02	0
Iodoacetamide	10 mg.	2.01	1.88	6.5
	25 mg.	2.01	1.87	7.0
Potassium permanganate	1 mg.	2.01	0	100

The results show that iodine and potassium permanganate inhibit the activity of the phosphatase, while iodoacetic acid and iodoacetamide, which are the specific inhibitors of urease and other enzymes containing thiol groups are ineffective. This shows that stronger oxidising agents in-activate the phosphatase. In this connection, it may be mentioned that Sizer (8) investigated the action of certain oxidants and reductants upon the activity of bovine phosphatase. He found that strong oxidants inactivate phosphatase by oxidising certain constituent amino acid like tyrosine but not tryptophane. Similarly pepsin (Herriott, 9) also is inactivated by the iodination of tyrosine in the protein molecule. Sizer (8) from his results suggests that the inactivation of phosphatase by strong oxidants such as iodine and potassium permanganate can be explained by the oxidation of substituent amino acid in the enzyme rather than by oxidation of sulphhydryl groups or by protein denaturation. The results obtained in the present investigation also conform to the same view.

*Effect of Nitrous acid.*

The following experimental mixtures were set up.

1. 20 cc. enzyme solution + 20 cc. acetate buffer (pH 5.2) + 20cc.  $H_2O$
  2. 20 cc. enzyme solution + 20 cc. acetate buffer (pH 5.2) + 20cc.,  $2MNaNO_2$
- and incubated at  $35^\circ C$  and 10 cc. aliquots were removed at zero time and at stated intervals, and after dialysis to remove traces of sodium nitrate, the activity was determined. As the nitrous acid interferes with the colorimetric estimation of phosphatase by its reaction with phosphomolybdous blue colour, it is necessary to remove the nitrate by dialyse. The results are presented in Table VI.

The results show that nitrous acid inactivates the phosphatase, the percentage inactivation increasing with increase in the time of incubation. This shows that amino group may be involved in the activity of the enzyme. Recently, Gould (10) investigating the significance of the amino and tyrosine groups in alkaline phosphatases (from kidney, bone and intestine) by deamination with nitrous acid reported that the activity is reduced by nitrous acid.

*Effect of formaldehyde.*

In view of the above observations on the effect of nitrous acid indicating the probable occurrence of amino group as one of the active groups of the enzyme, the influence of formaldehyde on the enzyme was investigated with a view to gather additional information on the occurrence of active amino groups in the enzyme molecule.

The following experimental mixtures were set up.

1. 20 cc. enzyme solution + 1 cc. formaldehyde + 9 cc.  $H_2O$ .
  2. 20 cc. enzyme solution + 10 cc.  $H_2O$ .
- and incubated at  $10^\circ C$  in a refrigerator. At stated intervals, 5 cc. of the solution were taken and the activity was determined after dialysis for 6 hours to remove formaldehyde. The results are presented in Table VI.

TABLE VI  
*Effect of sodium nitrite and formaldehyde on the leaf phosphatase.*

Reagent.	Concentration of reagent in experimental mixture.	Period of incubation.	Phosphatase activity.		Inactivation %
			Control.	Treated.	
Sodium nitrate	0.66 m	0	0.91	0.92	0
		5 min.	0.89	0.55	38.2
		15 "	0.91	0.37	59.8
		30 "	0.90	0.28	70.0
Formaldehyde	1.3 per cent.	0	0.91	0.91	0
		2 hrs.	0.91	0.75	17.5
		4 "	0.93	0.58	26.6
		8 "	0.88	0.52	42.0
		10 "	0.90	0.47	48.0

The results show that formaldehyde inactivates the enzyme, the percentage of inactivation increasing with increase in the time of incubation. The experiment also confirms the suggestion that the enzyme contains free active amino groups in

the enzyme molecule. Recently, Gould (10) also has shown that the alkaline phosphatases of kidney, bone and intestine are inactivated by formaldehyde, thereby indicating the presence of intact amino groups in the enzyme.

#### *Methylation with diazomethane.*

Diazomethane is a valuable methylating agent which is especially suitable for the methylation of phenolic and carboxylic groups. Further, it is a mild methylating agent, best suited for use with labile and sensitive substances as it reacts in neutral solution. It is usually used in solution in ether.

Diazomethane has been used for the methylation of phenolic groups in certain glucosides (Herzig and Schonbach, 11), starch (Schmidt and Zeutner, 12), ascorbic acid (Haworth and Hirst, 13), penicillin (Meyer, Hobby and Chaffee, 14); (Meyer, Hobby and Dawson, 15); (Hickey, 16) and (Cavallito, 17).

Very little is known regarding the effect of diazomethane on enzymes. Helferich, Specidel and Toeldte (18) have investigated the effect of Methylation by diazomethane on the activity of emulsin and observed that the enzyme was inactivated by treatment with diazomethane.

The influence of diazomethane on phosphatase activity has not yet been studied. The following experiments were, therefore, carried out with a view to study the effect of diazomethane on the purified leaf phosphatase.

*Preparation of diazomethane* (Owen, 19). Diazomethane was prepared in ether solution by adding ice cold aqueous potash and ether to the nitrosomethyl urea and distilling very slowly from a water-bath at about 50°C, until the distillate was no longer yellow. It was preserved in an ice chamber.

#### *Estimation of diazomethane in ether solution*

The diazomethane solution (Marshall and Acree, 20) was cooled and treated with an excess of ethereal solution of benzoic acid. The reaction takes place as indicated.

$C_6H_5COOH + CH_2N_2 \rightarrow C_6H_5COOCH_3 + N_2$ . The solution was then diluted with water and after the reaction is over the excess of benzoic acid was determined by titrating with N/10 sodium hydroxide, using phenolphthalein as the indicator, with vigorous shaking during titration.

The preparation contained 0.167 gram of diazomethane per 10 ml. of ethereal solution of diazomethane.

The following experimental mixture were set up.

1. 20 cc. of enzyme + 3 cc. of diazomethane in ether  
(containing 49.1 mg.  $CH_2N_2$ )
2. 20 cc. of enzyme + 4 cc. of diazomethane in ether  
(containing 65.8 mg.  $CH_2N_2$ )
3. 20 cc. of enzyme + 6 cc. of diazomethane in ether  
(98.2 mg.  $CH_2N_2$ )

and controls were run corresponding to each of the above experimental mixtures, using only ether without diazomethane. The mixtures were incubated at room temperatures (22-23°C). The contents were occasionally shaken and the methylation progressed, the  $CH_2N_2$  molecule in the ether breaks down which can be seen by the disappearance of the yellow colour from ether. The activity was determined

at stated intervals of time taking out aliquots of 10 cc. from the experimental mixtures. The  $pH$  of the experimental solutions was adjusted to  $pH$  5.2. The results are presented in Table VII.

The results show that the enzyme is inactivated by diazomethane, the percentage of inactivation increases with increase in the concentration of diazomethane. This shows that the activity of the enzyme is destroyed by methylation of the essential active groups of the enzyme. These may be either phenolic or carboxylic groups.

TABLE VII

*Influence of diazomethane on leaf phosphatase.*

Amount of diazomethane added mg.	Period of incubation (mins.)	Phosphatase activity in mg. P.		Inactivation per cent.
		Control.	Treated.	
49.1	30	2.67	1.9	28.8
	60	2.67	1.86	30.0
65.8	60	2.67	1.70	36.3
98.2	120	2.67	0.52	80.0

## DISCUSSION

Highly active preparations of phosphatase from french bean leaves have been obtained by adopting the procedure described above for the purification of the enzyme. The final product obtained possessed an activity of about 139 Ph. P.U./mg. which is about 160 times as active as the original leaf powder.

Chemical tests carried out on the purified enzyme preparations have given positive reactions for proteins and carbohydrates. Further studies on the chemical nature of the active groups of the enzyme have shown the presence of active amino and phenolic and hydroxyl groups in the enzyme molecule. This conclusion is based on the results of experiments on the influence of sodium nitrite formaldehyde and diazomethane on the enzyme. All these substances inactivate the enzyme. Sodium nitrite which as is well known deaminates amino acids, inactivates the enzyme possibly by deaminating the active amino group. In a similar manner, formaldehyde inactivates the enzyme by reacting with the active amino group of the enzyme. Inactivation by diazomethane which is a very sensitive methylating agent, shows the presence of active phenolic hydroxyl groups in the enzyme molecule, which on methylation renders the enzyme inactive. Further investigation, is however, necessary to throw more light on the chemical nature of the active groups in the enzyme molecule.

The study of the chemical nature of the active groups of enzymes has been mainly confined to investigation on the presence of thiol, amino and phenol hydroxylic groups in the enzyme molecule. The nature of the active groups in alkaline phosphomonoesterase has been the subject of study by some investigators. Williams and Watson (21) have studied the influence of sulphydryl compounds upon the activity of bone phosphatase and showed the sulphydryl group is not involved in phosphatase activity. Later, Sizer (8) from his studies on the action of certain oxidants and reductants upon the activity of bovine phos-

phatase (from kidney, lung and intestines) particularly by dilute iodine and permanganate concluded that the phosphatase activity is related to unaltered tyrosine in the molecule. This conclusion is based on the fact that the phosphatase is inactivated by dilute iodine and permanganate and that the ultraviolet absorption spectrum of phosphatase shifts to higher wavelengths, just as the absorption spectrum of tyrosine on the addition of strong oxidants. Recently, Gould (10) studied the chemical nature of the active groups in intestinal, bone and kidney phosphatases by treatment with ketene, phenyl isocyanate, nitrous acid and formaldehyde and showed that the intact amino group is essential for full enzymic activity. In this report, the phosphatase differs from pepsin (Herriott, and Northrop 22) whose activity is independent of the presence of amino groups. The present investigation in the chemical nature of the active groups in leaf phosphatase has thrown important light on the subject. The results obtained in the present investigation on the effect of sodium nitrite and formaldehyde are in conformity with the results obtained by previous workers on animal phosphatases. It does afford evidence to show that the activity of the phosphatase is dependent on the existence of free amino groups in the enzyme. The influence of diazomethane on the enzyme, has shown the existence of phenolic hydroxyl groups in the enzyme. Thus the leaf phosphatase is similar to the animal phosphatases which require amino and phenolic hydroxyl groups for their activity.

#### SUMMARY

1. Highly active preparations of leaf phosphatase were obtained by adopting the following method of purification.

1. fractional precipitation with alcohol.
2. solution of the most active fraction.
3. adsorption of the enzyme from the aqueous solution of the most active fraction at pH 3.6 by  $Al_2O_3 \cdot C\gamma$  gel.
4. Elution of the enzyme from the  $Al_2O_3 \cdot C\gamma$ -phosphatase adsorbate by 10 per cent sodium tartrate solution.
5. final dialysis.

By adopting the above method for the purification of the enzyme 140-160 fold increase in activity can be obtained. The final product possessed an activity of 139 Phy. P.U./mg. which is about 160 times as active as the original leaf powder.

2. The optimum pH of the purified leaf phosphatase was found to be 5.2.
3. The hydrolysis of sodium- $\beta$ -glycerophosphate by the leaf phosphatase obeys Michaelis and Menten's theory.
4. The order of increasing hydrolysability of substrates by the leaf phosphatase is as follows:

Pyrophosphate >  $\beta$ -glycerophosphate > Alpha glycerophosphate > Hexose-diphosphate.

5. Purified preparations of leaf phosphatase has been subjected to chemical tests and found to respond to proteins and carbohydrate tests.

6. The influence of papain, iodine, permanganate, iodoacetic acid, sodium nitrite, formaldehyde and diazomethane on the activity of the enzyme was investigated.



7. The chemical nature of the active groups in the enzyme molecule has been discussed in the light of the experimental results obtained on the influence of the above substances on the activity of the enzyme.

The authors' thanks are due to Professor V. Subrahmanyam for his interest in the above investigation.

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**ROLE OF VITAMINS IN THE METABOLISM OF IRON  
COPPER AND MANGANESE**

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The effect of different vitamins on the metabolism of calcium, magnesium and phosphorus has been reported in our previous communication from this laboratory (Basu and De, 1). The present paper deals with the study of the role of vitamins in the metabolism of other nutritionally essential elements as iron, copper and manganese by balance experiments on human subjects. A brief review of the work performed in other laboratories is given below.

### Vitamin A:

Occurrence of anæmia in cases of vitamin A deficiency is often reported in the literature. Although Josephs (2) in his review attributed this anæmia to infection accompanying vitamin A depletion, Mainer and Joel (3) in his experiments on anæmia in young children found rapid regeneration of red cells after vitamin A administration.

### B Vitamins:

Parsons *et al* (4), Day *et al* (5) and others have indicated the presence of an anti-anæmic factor in the vitamin B-complex by their study with synthetic diets on different animals. Some workers as Miller and Rhoads (6) and Hogan and Parrot (7) have claimed the above as one of the factors of the heat-stable fraction of the B-complex and this was indirectly supported by the investigation of Oldham *et al* (8, 9) who did not find any effect of vitamin B<sub>1</sub> in the retention of iron in rats in long term experiments.

### Vitamin C:

Association of anæmia with scurvy is frequent and there are evidences that this is due to defective iron utilisation when the body's reserve of ascorbic acid is depleted. Mettier *et al* (10) and Dunlop and Scarborough (11) reported prompt recovery from anæmia and marked blood regeneration after administration of ascorbic acid to the scorbutic subject.

### Vitamin D:

In contrast to the observations of Sure and Kik (12), Hauss (13), McDonough and Borgen (14) that vitamin D do not appear to affect hæmatopoietic function; Maughan (15) reported a reduction in the hæmoglobin level in the ricketic chicks and prompt rise of the level by ultraviolet irradiation of the animals and Day *et al* (16) noted greater degrees of anæmia on a vitamin D deficient diet containing an excess of phosphorus.

The above review indicates clearly that there are contradictory evidences regarding the influence of different vitamins on the production and cure of anæmia and retention of iron. The present work has been taken up to get more precise information about the subject by direct metabolic study on normal human adults.

## EXPERIMENTAL

The experimental subjects and technique of collection of urine and fæces were the same as that reported in our previous paper (Basu and De, *loc cit*). Iron was estimated by the thiocyanate method of Farrer (17), copper with diethyl dithiocarbonate reagent according to the method of Callan and Henderson (18) as modified by McFarlane (19) and Hoar (20) and manganese with potassium periodate reagent

by the method of Skinner and Peterson (21). Similar rice-fish (DI), wheat diets (DII) and vitamin supplements as were used in our previous communication were also employed in the present study.

## RESULTS AND DISCUSSION

**Vitamin A:** It is observed from the table I that administration of 1000 units of vitamin A daily improves the balance of iron to an appreciable extent. Its effect on copper and manganese is not conclusive and insignificant. The present work supports the observations of Mainzer and Joel (*loc. cit.*) who found rapid regeneration of red blood cell after vitamin A administration.

**Vitamin B-complex:** Administration of 10 g. of marmite (yeast product) with the daily diet (Table I) improved the retention of iron in both the subjects. The anti-anæmic effect of yeast as observed by Day *et al* (*loc. cit.*) and Hogan *et al* (*loc. cit.*) may be due to its beneficial effect on the utilisation of iron for normal hæmoglobin formation.

**Vitamin B<sub>2</sub>-Complex (heat-stable fraction of the B-complex):** Administration of marmite after destroying the heat labile factor (B<sub>1</sub>) of the complex by autoclaving, showed a promising effect on the retention of iron. The improvements in the retention of copper and manganese were also observed to a certain extent (Table II).

**Vitamin B<sub>1</sub>:** The supplementation of 6 mg. of vitamin B<sub>1</sub> 'Aneurin' with the basal diet (Table II) did not show any significant change in the metabolism of iron and copper. The most appreciable effect was observed in case of manganese, the retention of which was decreased by giving extra vitamin B<sub>1</sub>.

**Vitamin B<sub>2</sub> (Riboflavin):** The daily dose of 20 mg. of riboflavin along with the basal diets improved the balance of iron, copper and manganese to a considerable extent (Table III).

The beneficial effect of yeast in the utilisation of iron may be due to riboflavin or some other factor of the B<sub>2</sub>-complex. The anti-anæmic factor of B-complex as suggested by different workers seems to be elaborated by B<sub>2</sub> or some other factor of the heat stable fraction.

**Vitamin C:** The intake of 50 mg. vitamin C with the basal diet showed improvement in the retention of iron, copper and manganese (Table III). Anæmia associated with scurvy in vitamin C deficiency as observed by Mettier *et al* (*loc. cit.*) and Dunlop *et al* (*loc. cit.*) was presumably due to improper utilisation of iron and copper.

**Vitamin D:** Administration of 1000 units of vitamin D per day with the diet (Table IV) enhanced the retention of iron, copper and manganese by reducing their excretion. The occurrence of anæmia in rachitic rats as reported by Maughan *et al* (*loc. cit.*) and Day *et al* (*loc. cit.*) was most probably due to poor assimilation of the above elements on vitamin D deficiency. The impairment in the utilisation of iron due to deficiency of vitamin D is perhaps a secondary effect, the primary effect being in the absorption of calcium and phosphorus.

From the results presented in the above tables it seems clear that almost all the vitamins studied in the present investigation possess beneficial effect on the retention

TABLE I  
*Effect of Vitamin A on the metabolism of iron, copper and manganese—Daily dose 100 I/U.*  
*Vit. A content of basal diets: Subject G.C.N.—780 I/U. and Subject H.P.D.—710 I/U. per day.*

Vit. A content of basal diets: Subject G.C.N.—7801/U. and Subject H.P.D.—7801/V.														
Experimental subject	Diet	Supplement	Period	Iron metabolism.			Copper metabolism.				Manganese metabolism.			
				Dietary Fe (mg.) per day.	Urinary Fe (mg.) per day.	Faecal Fe (mg.) per day.	Balance (mg.) per day.	Dietary Cu (mg.) per day.	Urinary Cu (mg.) per day.	Faecal Cu (mg.) per day.	Balance (mg.) per day.	Dietary Mn (mg.) per day.	Faecal Mn (mg.) per day.	Balance (mg.) per day.
G.C.N.	D II	Nil	P II	47	0.47	38.53	+ 7.7	5	0.52	2.27	+ 2.2	7	4.49	+ 2.5
"	"	Vitamin A	Aver. of P III & P IV	47	0.69	32.85	+ 31.5	5	0.38	2.90	+ 1.7	7	4.83	+ 2.2
H.P.D.	D I	Nil	P II	35	0.62	29.18	+ 8.2	6	0.56	3.92	+ 1.5	12	8.74	+ 3.3
"	"	Vitamin A	Aver. of P III & P IV	38	0.79	20.15	+ 17.0	6	0.61	3.10	+ 2.3	12	6.12	+ 5.9
G.C.N.	D II	Nil	P II	41	0.68	33.34	+ 7.0	5	0.49	2.28	+ 2.2	6	3.58	+ 2.4
"	"	Marmite	Aver. of P III & P IV	41 + 9* = 50	0.52	16.27	+ 32.2	5	0.52	2.56	+ 1.9	6	3.98	+ 2.0
G.C.D.	D I	Nil	P II	48	0.71	36.12	+ 11.2	5	0.35	3.01	+ 1.6	10	5.72	+ 4.3
"	"	Marmite	Aver. of P III & P IV	48 + 9* = 57	0.75	30.08	+ 26.2	5	0.31	2.58	+ 2.1	10	4.12	+ 5.9
* The extra iron was incorporated along with Marmite. The copper and manganese contents of marmite were negligible.														

\* The extra iron was incorporated along with Marmite. The copper and manganese contents of marmite were negligible.

TABLE II

*Effect of B<sub>2</sub>-Complex (the heat stable fraction of the B-Complex) daily dose 10 g. autoclaved Marmite.*

Experimental subject.	Diet.	Supplement.	Period.	Iron metabolism.			Copper metabolism.				Manganese metabolism.			
				Dietary Fe (mg.) per day.	Urinary Fe (mg.) per day.	Faecal Fe (mg.) per day.	Balance (mg.) per day.	Dietary Cu (mg.) per day.	Urinary Cu (mg.) per day.	Faecal Cu (mg.) per day.	Balance (mg.) per day.	Dietary Mn (mg.) per day.	Faecal Mn (mg.) per day.	Balance (mg.) per day.
K.R.G.	D II	Nil	P II	60	0.60	54.1	+ 5.3	4	0.53	2.51	+ 1.0	7	5.56	+ 1.4
"	"	Autoclaved Marmite	Aver. of P III & P IV	60+9* =69	0.53	49.61	+ 18.8	4	0.40	1.49	+ 2.1	7	4.60	+ 2.4
G.C.N.	D I	Nil	P II	49	0.72	32.12	+ 9.2	5	0.40	3.28	+ 1.3	11	8.34	+ 2.7
"	"	Autoclaved Marmite	Aver. of P III & P IV	49+9* =58	0.74	31.08	+ 26.2	5	0.35	3.01	+ 1.6	11	7.12	+ 3.9
G.C.N.	D II	Nil	P II	39	0.61	33.91	+ 4.5	4	0.34	1.82	+ 1.8	13	8.28	+ 4.7
"	"	Aneurin	Aver. of P III & P IV	39	0.59	34.84	+ 3.5	4	0.39	1.68	+ 1.9	13	9.09	+ 3.9
G.C.D.	D I	Nil	P II	45	0.39	35.94	+ 8.7	5	0.28	3.45	+ 1.3	11	8.60	+ 2.4
"	"	Aneurin	Aver. of P III & P IV	45	0.45	34.18	+ 10.3	5	0.34	2.82	+ 1.8	11	10.45	+ 0.5

\* The extra iron was ingested along with Marmite. Copper and manganese supplied by marmite were negligible.

\* The extra iron was ingested along with Marmite. Copper and manganese supplied by marmite were negligible.

TABLE III

*Effect of Vitamin B<sub>2</sub> (Riboflavin)—daily dose 20 mg. riboflavin.*

Experimental subject.	Diet.	Supplement.	Period.	Iron metabolism.				Copper metabolism.				Manganese metabolism.			
				Dietary Fe (mg.) per day.	Urinary Fe (mg.) per day.	Faecal Fe (mg.) per day.	Balance (mg.) per day.	Dietary Cu (mg.) per day.	Urinary Cu (mg.) per day.	Faecal Cu (mg.) per day.	Balance (mg.) per day.	Dietary Mn (mg.) per day.	Faecal Mn (mg.) per day.	Balance (mg.) per day.	
K.R.G.	D I	Nil	P II	31	0.51	23.97	+ 6.5	4	0.60	2.20	+ 1.2	10	6.38	+ 3.6	
"	"	Riboflavin	Aver. of P III & P IV	31	0.50	8.94	+ 21.6	4	0.52	1.18	+ 2.3	10	6.12	+ 3.9	
G.C.N.	D II	Nil	P II	25	0.76	18.21	+ 6.0	5	0.51	3.58	+ 0.9	7	5.14	+ 1.9	
"	"	Riboflavin	Aver. of P III & P IV	25	0.68	10.36	+ 14.0	5	0.54	2.19	+ 2.3	7	3.09	+ 3.9	
H.P.D.	D I	Nil	P II	30	0.61	25.53	+ 3.9	6	0.38	2.15	+ 3.5	11	6.89	+ 4.1	
"	"	Ascorbic acid	Aver. of P III & P IV	30	0.56	8.32	+ 21.1	6	0.31	1.62	+ 4.1	11	5.07	+ 5.9	
K.R.G.	D I	Nil	P II	35	0.51	23.42	+ 11.1	4	0.21	3.16	+ 0.6	10	7.58	+ 2.4	
"	"	Ascorbic acid	Aver. of P III & P IV	35	0.59	18.18	+ 16.2	4	0.21	2.71	+ 1.1	10	5.32	+ 4.7	

TABLE IV

*Effect of Vitamin D—daily dose 1000 I/U.*

Experimental subject.	Diet.	Supplement.	Period.	Iron metabolism.			Copper metabolism.			Manganese metabolism.				
				Dietary Fe (mg.) per day.	Urinary Fe (mg.) per day.	Faecal Fe (mg.) per day.	Balance (mg.) per day.	Dietary Cu (mg.) per day.	Urinary Cu (mg.) per day.	Faecal Cu (mg.) per day.	Balance (mg.) per day.	Dietary Mn (mg.) per day.	Faecal Mn (mg.) per day.	Balance (mg.) per day.
G.C.N.	D I	Nil	P II	39	0.52	31.84	+ 6.5	4	0.42	2.81	+ 0.80	9	6.42	+ 2.6
"	"	1000 I/U Vitamin D	Aver. of P III & P IV	39	0.50	22.53	+ 16.0	4	0.36	2.01	+ 1.6	9	5.12	+ 4.9
H.P.D.	D I	Nil	P II	27	0.69	21.16	+ 5.1	6	0.34	3.00	+ 2.7	7	5.05	+ 2.0
"	"	1000 I/U Vitamin D	Aver. of P III & P IV	27	0.52	15.92	+ 10.6	6	0.39	1.66	+ 3.9	7	3.78	+ 3.2



of iron, copper and manganese. Significant improvement in the balance of copper and manganese was observed only in the cases of the vitamins B<sub>1</sub>, C and D. This improvement in the retention of iron, copper and manganese is effected by an increase in their absorption through the intestine as is evident from diminution in their faecal excretion. This increased absorption through intestine under the influence of the vitamins may be due to decrease in the pH of this organ as a result of which the precipitation of calcium phosphate and removal of soluble ions like iron, copper and manganese along with the insoluble calcium phosphate is retarded. The retardation of the individual precipitation of other phosphates also seem to occur.

### SUMMARY

1. Administration of the vitamins A, B-complex, B<sub>2</sub>-complex (heat stable fraction of the B-complex), B<sub>2</sub> (riboflavin), C and D to the rice and wheat diets improve the retention of iron to considerable extent. Vitamin B<sub>1</sub> was found to show an adverse effect in this respect.

2. Ingestion of the vitamins B<sub>2</sub>, C and D help also in the retention of copper and manganese to a certain extent.

3. The anti-anæmic factor of the B-complex as indicated by different workers seems to be elaborated either by riboflavin or some other factor of the B<sub>2</sub>-complex.

4. The improved retention under the influence of vitamins is due to enhanced absorption through intestine probably resulting from decrease in the pH of this organ.

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STUDY OF THE BISULPHITE BINDING SUBSTANCE OF URINE OF  
NORMAL HUMAN ADULT FED ON DIFFERENT RICE DIETS FOR  
ASSESSMENT OF THE VITAMIN B<sub>1</sub> LEVEL OF THE DIETS TO SATISFY  
THE ADULT REQUIREMENT

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Nutritional investigation of different Indian dietaries carried out in this laboratory by metabolic experiments on human subjects has shown that the ordinary rice diet is inadequate with regard to calcium and protein contents (Basu, Basak and De, 1). Different varieties of rice are consumed by the people of India living in different parts and various processes are generally adopted for their preparation from paddy. The various methods of curing of rice as are generally adopted in India influence their nutritive value to a considerable extent. The most notable effect is produced in the vitamin B<sub>1</sub> content, a major portion of which is wasted away when the rice in the raw condition is subjected to high polish. The process of parboiling which is generally practiced in the rural areas of India, leads to the conservation of a large percentage of this vitamin in rice even when it is highly polished afterwards. Washing and cooking for the preparation of the diet destroys about 50% of this vitamin.

The purpose of the present investigation is to study how far the rice diets prepared from parboiled home-pound and raw highly polished rice diets can satisfy the minimum requirement of vitamin B<sub>1</sub> for normal adult.

EXPERIMENTAL

On the basis of the observations of some workers (Collazo, 2 ; Bickel, 3, 4) that vitamin B<sub>1</sub> deficiency produces marked rise in the level of lactic and pyruvic acids and other bisulphite binding substances (B.B.S.) in urine and blood, Banerjee and Harris (5) and Harper and Deuel (6) developed a new technique for assessing the state of nutrition by determining the B.B.S. level of urine as an alternative to the measurement of vitamin B<sub>1</sub>. By a series of studies on rats and human subjects it has been shown that the level of B.B.S. in urine was proportional to the extent of deficiency of vitamin B<sub>1</sub>. The above technique of Banerjee and Harris has been adopted in the present investigation.

The composition of the diets prepared from two varieties of rice is shown in table I. At first the subjects G.C.N., H.P.D. and K.R.G. were fed on the parboiled

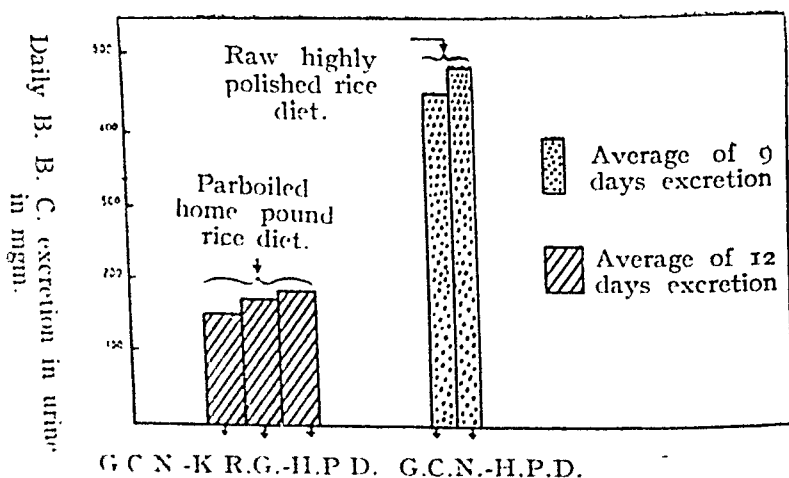
home-pound rice diet for a period of thirty-six days. Collection of urine was made on the last thirteen days of the experiment and their total bisulphite binding substance (B.B.S.) expressed in pyruvic acid was measured by the method of Clift and Cook (7). After completion of the above experiment with parboiled home-pound rice diet, the subjects G.C.N. and G.C.D. were then given raw highly milled rice diet for the same period as the previous experiment, collection of urine being made in this case on the last nine days of the experiment. Since K.R.G. suddenly left the laboratory, urinary B.B.S. study with this diet could not be performed on him.

TABLE I  
*Composition of the diets*

Name of the foodstuffs.	Parboiled home pound rice diet (g.)	Raw highly milled rice diet (g.)
Parboiled home-pound rice	550	×
Raw highly milled rice	×	550
Pulse	50	50
Fish	70	70
Potato	100	100
Brinjal	50	50
Green plantain	50	50
Mustard Oil	30	30
Total calories	2650	2650
**Vitamin B <sub>1</sub> content of the prepared diet	0.72 to 0.78 mg.	0.32 to 0.36 mg.

\*\* The loss due to washing and cooking was taken into account.

FIG. I



### RESULTS AND DISCUSSION

The results of the experiment are presented in table II and Fig. I from which it is observed that the B.B.S. excretion of urine of the three subjects G.C.N., H.P.D. and K.R.G. on parboiled home-pound rice diet ranged from 107 to 247 mg. and the average values of the three subjects were found to be 149, 168 and 180 mg. respectively with the mean value of 165 mg. The corresponding value on raw highly polished rice diet varied from 368 to 564 mg. and the average excretions produced by the subjects G.C.N. and H.P.D. on this diet were found to be 449 and 467 mg. respectively with the mean value of 467 mg. •

TABLE II

*Showing the Bisulphite Binding Substances (B.B.S.) in urine on parboiled home-pound and raw highly milled rice diets.*

B.B.S. expressed in mg. of pyruvic acid.

Days of Experiment.

Experimental diet.	Subject.	Days of Experiment.											Mean of the means.	
		25th day.	26th day.	27th day.	28th day.	29th day.	30th day.	31st day.	32nd day.	33rd day.	34th day.	35th day.	36th day.	Mean.
Parboiled home pound Rice diet.	G.C.N.	164	107	171	120	162	154	184	133	162	182	114	131	149
	K.R.G.	181	244	247	122	141	159	184	112	165	142	128	198	168
	H.P.D.	212	203	160	184	224	160	199	188	146	202	132	145	180
Raw highly milled rice diet.	G.C.N.				458	512	398	419	495	368	452	528	414	467
	H.P.D.				564	491	473	521	398	463	524	482	458	486

The B.B.S. values recorded in the present investigation corroborate with those of Banerjee and Harris (*loc cit*) who found higher B.B.S. values ranging from 470 to 670 mg. in urine of patients fed on beri-beri producing diet composed of highly polished rice. Harper (*loc cit*) and Waino (8) in their study of deficiency and requirement of thiamine in rats, observed similar increase in pyruvate excretion in urine on vitamin deficiency.

Table I shows that the difference in the two diets lies in the varieties of rice used which, however, produced considerable variation in the vitamin B<sub>1</sub> content of the diets. Since all other items and the total calorie supply of the two diets remained unaltered, it may be interpreted that the average difference of about 300 mg. of B.B.S. excretion on the two rice diets is due to difference in their vitamin B<sub>1</sub> contents.

#### TEST FOR DETERMINING THE VITAMIN B<sub>1</sub> ADEQUACY OF THE RICE DIETS TO SUPPLY THE MINIMUM REQUIREMENT OF HUMAN ADULT

It is observed from table I that the vitamin B<sub>1</sub> contents of parboiled home-pound rice diet and the raw highly milled rice diet varied from 0.72 to 0.78 mg. and from 0.32 to 0.36 mg. respectively. It was therefore, thought necessary to determine whether the above quantities of vitamin B<sub>1</sub> of the two rice diets could satisfy the minimum requirement for normal adult. To evaluate this the following load test was adopted.

After a preliminary period of 4 weeks on parboiled home-pound rice diet the urine of the subject G.C.N. was collected from the 19th to 21st days of the experiment and their B.B.S. were measured. This was followed by the daily supplement of 6 mg. of vitamin B<sub>1</sub> (Aneurin) along with the diet for six days—the urinary collection on this vitamin supplement being made on the last three days. The same experiment was conducted also with the raw highly polished rice diet.

TABLE III

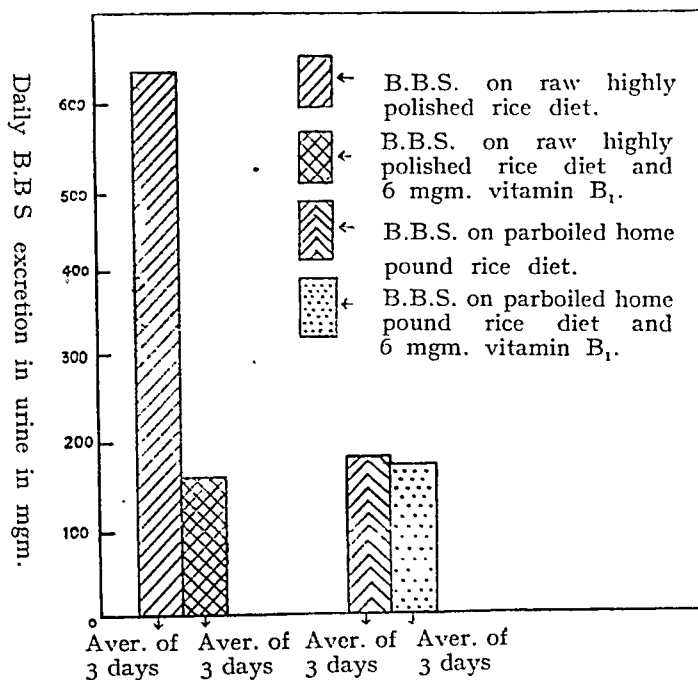
*Showing the effect of administration of extra Vitamin B<sub>1</sub> along with the two rice diets on the urinary excretion of B.B.S.*

Experimental Subject—G.C.N.

Supplement.	Days of experiment.	Urinary B.B.S. in mg. per day.	
		Raw highly polished rice diet.	Parboiled home-pound rice diet.
Nil	29th	699.4	173.7
	30th	559.3	202.6
	31st	656.4	195.3
Average		637.8	189.5
6 mg. Vitamin B <sub>1</sub> (Aneurin) per day	32nd	515.4	230.6
	33rd	450.0	156.3
	34th	180.0	130.6
	35th	100.2	270.5
	36th	163.2	121.1
	37th	228.1	157.4
Average of the last three days		163.9	183.0

It will be noted from table III and Fig. II that the administration of 6 mg. vitamin B<sub>1</sub> along with the parboiled home-pound rice diet did not produce any change in the B.B.S. excretion of the subject—the mean values before and after the vitamin supplement being 189.5 mg. and 183.0 mg. respectively. But most promising effect was observed when the above vitamin supplement was given along with the raw highly polished rice diet. In this case the average B.B.S. value dropped from 637.8 to 163.9 mg.

FIG. II



Since the B.B.S. excretion of human subject fed on highly polished rice diet suddenly dropped whereas that on parboiled rice diet remained unaltered after administration of extra vitamin B<sub>1</sub> to both the diets, it may be inferred that the vitamin B<sub>1</sub> content of the parboiled home-pound rice diet ranging from 0.72 to 0.78 mg. is sufficient to supply the adult requirement whereas that of the raw highly polished rice diet ranging from 0.32 to 0.36 mg. is below the requirement for carrying out the normal metabolic activities in the body.

Holt (9) in his review on "Thiamine requirement of man" indicated that the average intake between 0.24 to 0.44 mg. per 1000 calories appeared to be protective against thiamine deficiency. On the basis of this calculation the two rice diets used in the present investigation with calorie yields of 2650, should contain at least 0.63 mg. of thiamine for protection against deficiency. Aykroyd *et al* (10) in his review on "The rice problems of India" have shown that typical parboiled rice diet as consumed in India, with calorie yields ranging from 2000 to 3000 contain 660 to 900 micrograms of vitamin B<sub>1</sub>—the quantity which is required to prevent beri-beri. It is evident from table I that the vitamin B<sub>1</sub> of the parboiled home-pound rice diet is above whereas that supplied by the raw highly polished rice diet is below the minimum limit of 0.63 mg. as recommended by both Holt and Aykroyd and the adult requirement of this vitamin evaluated in the present investigation by study of the urinary B.B.S. excretion agrees well with this recommendation.

Since the B.B.S. excretion of human subjects fed on parboiled home-pound rice diet ranged between 100 to 300 mg., this limit may be regarded as indicative of protection against deficiency. The excretion values above the limit of 300 mg. will on the contrary indicate deficiency.

### SUMMARY

(1) The daily urinary excretion of the bisulphite binding substance (B.B.S.) of normal adult fed on parboiled home-pound rice diet ranged from 107 to 247 mg. whereas that on raw highly polished rice diet from 368 to 699 mg.

(2) By load test it has been found that ingestion of 6 mg. of extra vitamin B<sub>1</sub> with the highly polished diet brought down the average value of B.B.S. from 637.8 to 272.7 mg. No such change was observed in the case of home-pound rice diet. By this test it may be inferred that the range of 0.72 to 0.78 mg. vitamin B<sub>1</sub> supplied by the parboiled home-pound rice diet is above whereas the content of 0.32 to 0.36 mg. supplied by the raw highly polished rice diet is below the minimum limit for protection against deficiency.

(3) The daily B.B.S. excretion of normal adult above 300 mg. will indicate deficiency of vitamin B<sub>1</sub> whereas the value below this limit will represent the dietary conditions satisfying the requirement of this vitamin for normal Indian adult.

### ACKNOWLEDGEMENT

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**STUDIES ON THE PREPARATION OF ASCORBIC ACID FROM THE  
INDIAN GOOSEBERRY (*PHYLLANTHUS EMBLICA*)**

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In view of the widespread occurrence of ascorbic acid in Plant Kingdom and its rapid deterioration during storage, numerous methods have been developed for its isolation from plant sources either as active concentrates or crystalline product. Svirbely and Szent-Gyorgi (1) developed a method for its isolation from



Hungarian Paprica (*Capsicum annum*), which involved the precipitation of the vitamin as lead salt, removal of lead by using a mineral acid, extraction of the vitamin by organic solvents and subsequent concentration at reduced pressure. This procedure was later modified by Banga and Szent-Gyorgi (2) for the preparation on a large scale.

Many investigators, notably Zilva (3), Bessenoff (4) and King *et al* (5, 6) succeeded in obtaining very active concentrates of ascorbic acid starting from lemon juice. Later, Waugh and King (7) were able to obtain crystalline ascorbic acid from lemon juice. Starting with an aqueous concentrate of Dogrose fruits Schmidt and Tulchinskaya (8) prepared crystalline ascorbic acid by alternate extraction with alcohol and ether or acetone and concentration in vacuo. Yamamoto and Takeshi Hara (9) observed that magnesium oxide forms a compound with ascorbic acid, while preparing active concentrates from Pineapple juice.

An extensive literature (10, 11) exists indicating the Indian Gooseberries (*Phyllanthus emblica*) as one of the richest sources of ascorbic acid among Indian foodstuffs. Its ascorbic acid content is 2.0-2.5 g. in 100 g. of the dried and seed-free material. While the vitamin is very well protected in the fruit, it rapidly deteriorates during drying and storage at the ordinary temperature. Besides ascorbic acid, the gooseberry contains a large percentage of tannins, mucilages, resinous matter etc. It was thought desirable, therefore, to isolate ascorbic acid free from most of these interfering substances which may contribute to its destruction during storage. The adsorption technique using magnesium oxide developed by Sastri and Shivaramakrishnan (12) has been further investigated and the results are presented in this paper.

## EXPERIMENTAL

### *Preparation of Material.*

Mature berries are collected and dried in the sun as quickly as possible (about four days). The seeds are then removed using wooden hammer, the seed-free material well powdered and stored in airtight containers in the ice room. It can be stored thus for short periods without loss of vitamin.

During drying in the sun some loss of ascorbic acid occurs and it can be reduced to a minimum if the drying is carried out in the shade.

### *Extraction of Ascorbic acid.*

By trial it has been found that ascorbic acid is quantitatively extracted from the powder by shaking with aldehyde-free alcohol for two hours, the solid liquid ratio being 1:10. Along with ascorbic acid a good percentage of tannins and

resinous matter are also extracted. This alcoholic extract keeps without loss of ascorbic acid for long periods when stored in the ice room. This extract is used for the succeeding experiments.

### *Estimation of ascorbic acid.*

Ascorbic acid is estimated by titrating the test solution in trichloroacetic acid medium with 2,6 dichlorophenol indophenol reagent standardised according to the method of Meneker and Guerrant (13). A careful check is maintained on the strength of the dye solution. Glass-distilled water is employed throughout the work.

### *Adsorption.*

With a view to select a suitable adsorbent for ascorbic acid in alcoholic medium the following experiment is undertaken using a number of common adsorbents.

10 cc. of alcoholic extract containing 20 mg. of ascorbic acid are shaken with 0.5 mg. of adsorbent for about three minutes in a centrifuge tube. The mixture is then centrifuged and the vitamin content of the supernatant estimated by titrating an aliquot with standard dye solution. The quantity of ascorbic acid adsorbed is calculated by difference.

It is found that while light magnesium oxide is a very good adsorbent for ascorbic acid, substances like zinc oxide, calcium hydroxide, fibrous alumina, clarite, litharge, starch etc. are relatively poor. When light magnesium oxide is previously heated for a few hours at 700°C, its adsorptive power is enhanced to a remarkable extent and the adsorption becomes almost quantitative. Calcined magnesium oxide is therefore selected as the best adsorbent for ascorbic acid.

### *Influence of pH on Adsorption.*

The pH of 5 cc. of the alcoholic extract is adjusted by adding N/10 oxalic acid and the volume of the liquid made up to 10 cc. with water. After thorough mixing and allowing sufficient time for the system to attain equilibrium, the pH is measured using the glass electrode. The 0.5 g. of magnesium oxide is added, shaken thoroughly and centrifuged. The ascorbic acid unadsorbed is estimated and hence the amount adsorbed is calculated. The experiment is repeated at different pH using varying quantities of oxalic acid and sodium carbonate.

That the adsorption of ascorbic acid is greatly influenced by the presence of other substances in the alcoholic extract is shown by repeating the experiment in exactly the same manner using standard ascorbic acid solution in alcohol. The results are tabulated in Tables I and II.

TABLE I

*Influence of pH on adsorption.*

5 cc. of alcoholic extract; 5 cc. standard ascorbic acid solution used. In the former case pH adjusted by the addition of N/10 oxalic acid; N/10 sodium carbonate and in the latter by Mcllvaine's buffer.

Treatment	pH	Adsorption %
(a) Oxalic acid.		
(1)	3.01	84.3
(2)	4.53	81.3
(3)	2.62	74.6
(4)	2.93	69.7
(b) Sodium carbonate.		
(1)	3.60	100.0
(2)	4.61	66.7
(3)	5.69	61.6
(4)	5.58	60.1
(c) Mcllvaine's buffer.		
(1)	3.54	61.0
(2)	4.26	70.3
(3)	4.65	100.0
(4)	4.78	80.1
(5)	5.17	86.2
(6)	6.32	70.0
(7)	8.07	68.0

It is clear from the above results that while the optimum pH in the case of pure ascorbic acid solution lies near 4.6, that for alcoholic extract is 2.8-3.0.

*Optimum quantity of the adsorbent.*

For economic working it becomes absolutely necessary to determine the minimum quantity of adsorbent required to achieve the maximum adsorption. It is evident from Table II, in which is shown the change in the adsorption with varying quantities of magnesium oxide, that 10 g. of adsorbent for 100 cc. of the alcoholic extract gives the maximum adsorption.

TABLE II

*Influence of quantity of MgO on adsorption of Ascorbic acid.*

10 cc. of alcoholic extract containing 24.7 mg. of ascorbic acid are used.

Wt. of MgO used	Adsorption %
0.2	9.8
0.5	55.2
0.75	77.9
1.0	94.8

*Influence of Temperature on Adsorption.*

It is observed that there is considerable destruction of ascorbic acid when adsorption is carried out at room temperature, as judged from the recovery of the vitamin after elution. Owing to the basic nature of the magnesium oxide and the presence of other interfering substances in the medium ascorbic acid undergoes rapid oxidation during adsorption. This process of oxidation is reduced to a minimum when the adsorption is carried out at  $0^{\circ}\text{C}$ , the alcoholic extract being cooled in ice before the addition of the adsorbent. (*Vide* Table III).

TABLE III

*Influence of Temperature on adsorption.*

(The results are averages of at least five determinations in each set.)

Temperature of Adsorption	Elution %		Destruction %
	CO <sub>2</sub> and Water	H <sub>2</sub> S and Water	
	Before reduction with H <sub>2</sub> S	After reduction with H <sub>2</sub> S	
$0^{\circ}\text{C}$	31	70	39
$0^{\circ}\text{C}$	—	—	29.8
Room temperature			
( $25^{\circ}\text{C}$ )			
(i)	29	Nil	71
(ii)	—	—	49

*Elution.*

To 150 cc. of alcoholic extract in a round bottom flask cooled in ice are added 15 g. of magnesium oxide with stirring. The flask is kept corked in the ice-bath with occasional shaking for nearly thirty minutes. The mixture is then centrifuged, the adsorbate suspended in sufficient volume of water and elution under different conditions of temperature and time are tried. In each case the eluate is filtered or centrifuged, made up to a known volume and an aliquot used for the estimation of ascorbic acid.

It is observed that dehydroascorbic acid is formed during the process of elution and the irreversible oxidation of dehydroascorbic acid is quickened depending upon the temperature and time of heating. Elution is possible by using carbon dioxide but due to the formation of dehydroascorbic acid, hydrogen sulphide is found to be most suitable. The results obtained further showed that the oxidation of ascorbic acid to dehydroascorbic acid in contact with magnesium oxide is little affected by temperature ; but the irreversible oxidation which takes place rapidly at higher temperatures is considerably reduced when the adsorption is carried out at  $0^{\circ}\text{C}$ .

### Method

Thus having standardised the various conditions for adsorption and elution, the following simple procedure has been evolved for the preparation of ascorbic acid concentrate.

The gooseberry powder is extracted with aldehyde free alcohol by shaking for two hours. The clear extract obtained is cooled in ice and light magnesium oxide added with vigorous stirring to make a final concentration of ten percent. The flask is tightly stoppered and kept in the ice bath for nearly thirty minutes with occasional shaking to maintain the magnesium oxide in suspension. The mixture is centrifuged. The adsorbate is transferred quickly to a flask, suspended in sufficient volume of water saturated with hydrogen sulphide and centrifuged. This is repeated and the eluates are mixed, saturated with hydrogen sulphide and left overnight. A small quantity of oxalic acid is added and the precipitate formed filtered off. The clear liquid is concentrated to a small volume under reduced pressure at 25°C. The concentrate is filtered to remove any precipitate formed. The clear light reddish-yellow liquid thus obtained is stored in amber coloured bottles.

Experiments conducted using large quantities of the extract according to the above procedure give uniform yield as shown in Table IV.

TABLE IV

Volume of Extract (cc.)	Ascorbic acid before treatment (mg.)	Ascorbic acid in the concentrate (mg.)	Yield %
500	1200	804	67.0
1000	2400	1600	66.7
2000	4780	3060	64.0
3000	7050	4450	63.0

The concentrate obtained is entirely free from tannins and most of the resinous and mucilaginous substances. It has an agreeable taste.

The loss of vitamin is negligible at room temperature when the concentrate is preserved with sugar and stored in amber coloured bottles.

Crystals of ascorbic acid are obtained from the concentrate by extraction with mixtures of methanol, acetone and anhydrous ether.

### DISCUSSION

The preparation of ascorbic acid from the press juice of Indian gooseberries according to the method outlined by Svirbely and Szent-gyorgi (1) results finally in a sticky mass. There is also considerable loss of vitamin during each precipitation with lead acetate; further the tannins, mucilages and resinous substances are not removed to a great extent. With the alcoholic extract which excludes mucilages it is found that though about 60% of the tannins could be removed avoiding appreciable loss of vitamin by precipitating with saturated solution of basic lead,

the resulting liquid on concentration gives a deeply coloured concentrate having a characteristic taste. Crystallisation of ascorbic acid from this concentrate is found to be very difficult. In the adsorption method developed these disadvantages are overcome. Further it has the following advantages:—(1) it is very simple and does not involve costly chemicals; (2) the yield is on the order of 65%; (3) the tannins responsible for the astringent taste are completely removed; (4) other resinous substances and most of the colouring matter are also removed; (5) the concentrate obtained is palatable and the crystallisation easy; and (6) the adsorbent once used can be recovered by calcining at high temperature and used again.

In the alcoholic medium, magnesium oxide serves as a good adsorbent as it is almost insoluble in alcohol. As the  $pH$  of the alcoholic extract is in the vicinity of the  $pH$  optimum for adsorption it can be used without further treatment.

When carbondioxide is used during elution, it is found that 30% of the vitamin occurs in the reduced state and an additional yield of 40% can be recovered by further treatment with hydrogen sulphide. This double treatment can be avoided by the use of hydrogen sulphide when the total yield of 70% is obtained directly as ascorbic acid. With the increase in temperature, the rate of irreversible oxidation of ascorbic acid is enhanced making it absolutely essential to keep the temperature of the medium at  $0^{\circ}C$  during adsorption.

The results given in Table IV indicate the possibility of this method being used for the preparation of ascorbic acid on a large scale. With the aid of proper equipment for adsorption and concentration in vacuo, concentrates of any desired potency can be obtained.

#### SUMMARY

Experiments with alcoholic extract of the Indian gooseberries showed that calcined magnesium oxide serves as an excellent adsorbent for ascorbic acid.

The influence of  $pH$  of the medium, quantity of adsorbent, temperature and methods of elution have been studied. While it is possible to use either carbon dioxide or hydrogen sulphide for elution, the latter is to be preferred as about 40% of vitamin exists as dehydroascorbic acid in the eluate.

A simple and economical method has been evolved for the preparation of ascorbic acid from the Indian gooseberry. The concentrate obtained is free from tannins and most of the resinous and colouring matter and can be preserved with sugar.

The advantages of this method and its possible application for large scale preparation of ascorbic acid are discussed.

The author is indebted to Mr. B. N. Sastri and Dr. Y.V.S. Rau for their helpful suggestions and Prof. V. Subrahmanian for his keen interest and encouragement throughout the course of the investigation. His thanks are also due to the Madras Government for the grant of a scholarship.

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EFFECT OF ASCORBIC ACID AND NICOTINIC ACID ON THE URINARY  
EXCRETION OF SUGAR BY ALLOXAN-DIABETIC RATS

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It has been shown by Banerjee and Ghosh (1) that deficiency of ascorbic acid in guinea pigs leads to a change in the carbohydrate metabolism as manifested by (a) glycosuria, (b) diminished tolerance to glucose, (c) lower glycogen content of the liver and (d) diminished insulin content of the pancreas. It has been suggested

that ascorbic acid is in some way related with the secretion of insulin in the pancreas.

Nicotinic acid has also been reported to have some effect on the carbohydrate metabolism. It has been observed by Gobell (2) that intravenous injection of nicotinamide in children produces a regular fall in the blood sugar level. Similar action of nicotinic acid has also been reported in normal human adults by Marche and Delbarre (3), Poumean-Delille and Fabiani (4) and Neuwahl. Neuwahl (5) has observed that nicotinic acid improves sugar tolerance and reduces the insulin requirement of diabetic patients. In non-diabetic subjects, reports the same author, intravenous injection of nicotinic acid produces blood sugar level as low as 48 mg. per 100 cc. which led to long lasting rigor. It has been suggested that nicotinic acid may be concerned in some way with factors which activate insulin. Hypoglycæmic effect of intravenous injection of large doses of nicotinic acid in rats has been reported by Clerks and Rosenfeld (6).

A study of the relation between these two vitamins and the urinary excretion of sugar of rats made diabetic by injection of alloxan therefore would be useful.

It has been shown by Musulin *et al* (7) that rats fed chloretone excrete a large amount of ascorbic acid in the urine. Chloretone fed rats synthesise excessive amount of ascorbic acid. It was therefore interesting to study the effect of injection of alloxan on the urinary excretion of ascorbic acid and sugar in the chloretone fed rats.

## EXPERIMENTAL

(a) *Effect of ascorbic acid and nicotinic acid on the urinary excretion of sugar in alloxan diabetic rats:* Healthy young rats of weight varying between 70 and 100 g. maintained on milk and whole wheat ration, were given intra-peritoneal injection of a 2 p.c. solution of alloxan (100 mg. per kilo). From the 2nd day of the experiment, the rats began to excrete sugar in the urine. Each rat after alloxan injection was placed in a metabolism cage and the urine was collected over 1 cc. of glacial acetic acid. The rats were divided into two groups. One of the groups received from the second day an intraperitoneal injection of 50 mg. ascorbic acid dissolved in 1 cc. of water. The second group of rats received similarly a daily dose of 10 mg. of nicotinic acid dissolved in 1 cc. of water. 24 hours urine was collected and 24 hours urinary excretion of sugar was estimated with Benedict's quantitative reagent. In order to see the effect of the vitamins in severe diabetes, some of the rats mentioned in the tables were given a second injection of alloxan. Results are given in Tables I and II.

(b) *Effect of injection of alloxan on the urinary excretion of ascorbic acid and sugar by chloretone fed rats:* Each rat was fed a daily dose of 20 mg. chloretone dissolved in 0.1 cc. of olive oil. When the rats began to excrete increased amount of ascorbic acid in the urine (third to fifth day after they were fed chloretone) each rat was given an intraperitoneal injection of alloxan (100 mg. per kilo).

The urinary excretion of ascorbic acid was estimated by titration against a standardised solution of 2:6-dichlorophenol indophenol. The results are given in Table III.

Another group of rats which serve as controls received only the injection of alloxan. Urinary excretion of sugar of these rats are given in Table IV.

TABLE I

*The effect of ascorbic acid on the urinary excretion of sugar by alloxan diabetic rats.*

(The rats received injections of alloxan (100 mg. per kilo) on the first day and on the 6th day of the experiment);

No. of rats.	24 hours urinary sugar (mg.)									
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day
1.	0	48	23	29	18	28	128	390	723	Dead
2.	0	44	40	12	20	18	149	480	1243	Dead
4.	0	25	23	28	13	34	49	580	1508	Dead
5.	0	43	19	17	25	20	48	428	562	920
6.	0	39	28	22	26	27	82	50	42	72

TABLE II

*The effect of nicotinic acid on the urinary excretion of sugar by alloxan diabetic rats.*

(Each of the rats received a single intraperitoneal injection of alloxan (100 mg. per kilo) on the first day of the experiment. The rats no. 12, 13 and 14 received a second injection of alloxan on the fifth day of the experiment).

No. of rats.	24 hours urinary sugar (mg.)										
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day	11th day
9.	0	49	26	20	15	18	18	20	22	13	31
10.	0	75	38	23	21	15	12	12	21	26	18
11.	0	51	38	21	21	45	—	23	31	61	42
12.	0	28	32	26	84	110	450	637	711	Dead	—
13.	0	10	20	16	59	330	758	761	Dead	—	—
14.	0	43	40	31	51	265	628	610	Dead	—	—

TABLE III

*Twenty-four hours' urinary excretion of ascorbic acid and sugar expressed in mg. in chlorotone fed rats after injection of alloxan.*

(The rats were fed chlorotone for five days and then they received two injections of alloxan on the 0 day and on the ninth day)

No. of rats	Day after injection of alloxan.													
	0	1	2	3	4	5	6	7	8	9	10	11	12	14
15 ascorbic acid	9.1	2.0	7.6	5.8	5.9	5.9	5.4	11.8	12.5	15.6	7.9	11.5	5.8	Dead
Sugar	—	29	39	36	45	45	26	68	51	66	182	348	720	Dead
16 ascorbic acid	—	4.5	3.5	4.6	2.9	1.2	1.2	4.2	5.9	6.8	7.5	—	8.4	Dead
Sugar	—	32	20	36	33	13	13	37	41	56	—	72	300	Dead
17 ascorbic acid	7.0	4.0	7.0	2.1	2.5	2.5	10.5	6.5	8.0	10.4	3.2	5.2	Dead	
Sugar	—	25	40	32	24	24	28	25	29	33	348	528	Dead	
18 ascorbic acid	4.2	3.4	7.3	4.5	2.2	2.2	24.4	2.8	10.4	8.2	8.3	9.4	4.2	Dead
Sugar	—	31	25	43	24	24	42	62	25	—	64	248	750	Dead
19 ascorbic acid	10.4	8.8	3.3	4.2	4.5	4.5	2.6	2.2	2.9	5.2	8.4	7.2	6.8	Dead
Sugar	—	18	42	48	22	22	19	30	26	22	258	584	1004	Dead
20 ascorbic acid	2.2	7.2	6.8	2.4	5.1	5.1	5.3	5.4	5.2	3.8	5.6	4.4	Dead	
Sugar	—	23	36	36	12	12	42	42	26	58	147	982	Dead	

TABLE IV

*Twenty-four hours' urinary excretion of sugar expressed in mg. by alloxan diabetic rats.*

(Each of the rats received a intraperitoneal injection of alloxan on the 0 day. The rats no. 21 and 22 received a second injection of alloxan on the fifth day and rats no. 23, 24 and 25 on the 9th day of the experiment).

No. of rats	Day after injection of alloxan.										
	0	1	2	3	4	5	6	7	8	9	10
21	0	45	40	32	22	28	133	682	892	Dead	
22	0	28	49	26	22	20	258	742	Dead		
23	0	44	33	30	13	13	57	—	—	43	333
24	0	22	19	18	38	23	—	42	—	18	89
25	0	32	—	42	24	24	31	24	26	—	96

## DISCUSSION

It could be seen from Table I, that urinary excretion of sugar in alloxan diabetic rats is not lowered by the injection of ascorbic acid. Banerjee (8) working with monkeys reported that injection of alloxan damages the  $\beta$ -cells of pancreas which are responsible for the secretion of insulin. It has also been reported by Banerjee and Ghosh (1) that in guinea pigs, ascorbic acid and synthesis of insulin in the body bears some specific relation and diminished insulin content of the pancreas of scorbutic guinea pigs is not merely due to the lowered vitality of the tissues of the scorbutic animals. It is unnatural therefore to expect that synthesis of insulin could be restored by the injection of ascorbic acid once the  $\beta$ -cells of pancreas responsible for secretion of insulin has been damaged by the injection of alloxan. The present work, thus is in no way contradiction to the findings of Banerjee and Ghosh (1). Furthermore investigations of Banerjee *et al* were carried out on guinea pigs which fail to synthesise ascorbic acid in the body while the present work deals with rats which possess the peculiar property of synthesising ascorbic acid in the body.

Nicotinic acid also seems to have no effect in lowering the urinary excretion of sugar by alloxan-diabetic rats. It is difficult to explain why nicotinic acid failed to have any response in diabetic rats, though it has been observed by Clerks and Rosenfeld (6) that injection of nicotinic acid into rats produced hypoglycemia. Findings of other authors (2, 3, 4, 5) can be explained by saying that results obtained while working with human subjects are not in all instances reproducible in rats which are the experimental animals in our present investigation

From the Table III, it could be seen that injection of alloxan into chlorotone fed rats excreting increased amount of ascorbic acid produces diabetes. Excretion of ascorbic acid in the urine of chlorotone fed rats is also not affected by the development of diabetes.

## SUMMARY

1. The effect of injection of 50 mg. of ascorbic acid in rats made diabetic by injection of alloxan (100 mg. per kilo) was studied. Ascorbic acid did not lower the urinary excretion of sugar nor did it prevent death from diabetes.

2. Intraperitoneal injection of 10 mg. of nicotinic acid in rats made diabetic by injection of alloxan failed to bring about any lowering of urinary excretion of sugar. Deaths from alloxan diabetes could not be prevented by regular injection of nicotinic acid.

3. Effect of injection of alloxan on chloretone fed rats excreting increased amounts of ascorbic acid in the urine was studied. The chloretone fed rats developed diabetes as manifested by the urinary excretion of sugar. Development of diabetes in chloretone fed rats failed to bring about any permanent lowering of urinary excretion of ascorbic acid.

4. Both ascorbic acid and nicotinic acid cannot restore the normal carbohydrate metabolism in rats made diabetic by injection of alloxan. Biosynthesis of ascorbic acid in chloretone fed rats is not affected by injection of alloxan. Biosynthesis of ascorbic acid in chloretone fed rats does not seem to be related with the secretion of insulin from the pancreas.

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INVESTIGATIONS IN PHYTONCIDES OR PLANT BACTERICIDES  
PART I. STUDIES IN THE ISOLATION AND ANTIBACTERIAL  
PROPERTIES OF THE PHYTONCIDE IN GARLIC  
(*ALLIUM SATIVUM*)

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Plants have for long been known to be useful in the treatment of infectious diseases. Tokin (1), while carrying on experiments with the effect of plant emanations on micro-organisms, found that a paste prepared from a small amount of macerated garlic, onion, or allied plants immediately emits volatile substances which are lethal to almost all protozoal and bacterial cultures. To such volatile substances produced by plants in their life cycle are endowed with strong bactericidal and protistocidal properties, Tokin gave the name 'Phytoncides'. Toroptsev and Filatova (2) claimed garlic and onion to contain the most powerful bactericidal properties of all the plants studied. Tokin and co-workers (1) claimed that only the emanations volatilizing out of crushed garlic cells are active against bacteria and protozoa and an exposure for 30 minutes in air completely deprives the garlic cells of their active substances. Uemori (3) and Dittmar (4) attributed the antibacterial activity of garlic extracts to diallyl sulphide, while Kitavawa and Amano (5) suggested that the antibacterial activity in garlic is mainly due to the unstable sulphur in alkyl polysulphides. Cavallito and Bailey (6) have shown that the oil of garlic obtained by steam distillation of crushed cloves and subsequent fractionation following the method of Semmler (7), when tested by the cylinder plate method, did not show any antibacterial activity, proving thereby that the natural diallyl disulphide



and diallyl polysulphide to be antibacterially inactive. Cavallito *et al* (6) have also isolated from garlic an antibacterial substance, named as allicin, which is claimed to be not so volatile and unstable as the Phytoncides are generally supposed to be by workers like Tokin, Filatova and Toroptsev (1, 2). Rao, Rao and Venkatraman (8) have also isolated allicin from garlic following a method different from that of Cavallito *et al* (6), but more or less confirming their observations. Allicin, according to Cavallito, is a colourless oily liquid, 2.5% soluble in water at 10°C, miscible with alcohol, benzene, ether and quickly inactivated by heat and alkalis. Allicin has been shown to be active against gram-positive and gram-negative organisms at a dilution of 1: 125,000 and it exerts a more bacteriostatic than bactericidal action.

Allicin has been given the structure allyl- $\overset{\text{S}}{\parallel}$ -s-allyl with the structure allyl-s-o-s-allyl  
 $\text{O}$   
 not entirely eliminated, after a thorough study by Cavallito, Buck and Suter (9) of its molecular weight, alkaline hydrolysates, molecular refractivity, adsorption spectra and polarographic measurements. Rao *et al* (8) have studied the antibacterial and antifungal properties of allicin, the effect of artificial gastric juice and intestinal fluids, effect of blood on allicin and also the effect of allicin on certain SH enzymes. They also have studied the effect of allicin on M. tuberculosis.

Since garlic has been claimed by so many workers to contain so powerful an antibacterial substance, the isolation of the active principle with all precautions to its volatile nature was tried and its antibacterial properties *in-vitro* and *in-vivo* were studied. In the present communication, we have stated only the preliminary observations regarding the nature of the substance and have worked out a method for isolation of the active substance which has been found to be active against gram-positive and gram-negative organisms at a dilution of 1 in 100,000 and the effect has been found to be more bactericidal than bacteriostatic.

### EXPERIMENTAL

Assuming that the phytoncide in garlic is highly volatile the experiments of Tokin were verified using the volatile emanations of garlic by adopting the following procedure.

A vacuum dessicator is thoroughly cleansed, washed with ether and subjected to vacuumization for 15 minutes at 20-30 mm. pressure. The dessicator is connected to a U-tube placed in cold water bath and the U-tube connecting to the vacuum suction through a long narrow iron-tubing. This arrangement is made to sterilize the air going into the dessicator. Then, at the bottom of the dessicator, minced garlic cloves are placed and on the porcelain disc slides containing drops of living bacterial or protozoal emulsion are placed. The dessicator is closed and suction is employed. The vapour of garlic fills the dessicator. Suction is discontinued after 1 minute. The microbes are exposed to the fumes for 10 minutes and then examined for the lethal effect by culturing the organisms taken from the slide. The experiment was done with the bacterial cells like *Staphylococcus aureus*, *Streptococcus hemolyticus*, *B. coli* and *Bact. typhosum*. The volatile emanations of garlic was found to prove lethal against *Staphylococcus aureus* and *Streptococcus hemolyticus* but not against *B. coli* and *Bact. typhosum*.

Next, attempts were made to extract the antibacterial principle from garlic by solvents. Garlic cloves were crushed and treated with alcohol, the alcoholic extract was filtered and it was then concentrated under vacuum. The residue left was desiccated at room temperature. The mass remaining was weighed and dissolved in sterile redistilled water to make a solution of 1 in 1000. The extract was tested for antibacterial activity by following the serial dilution technique. Extracts of ether, chloroform, acetone and petroleum ether were made in the same manner as in the case of alcoholic one and each of them was dissolved in sterile redistilled water to a dilution of 1 in 1000 and tested for antibacterial activity by following the same technique. The results of solvent extraction procedures with garlic are summarised in Table I.

Fractions of garlic.	Bacteria used.	Results.	Concentration
1. Alcohol soluble fraction.	<i>B. coli</i>	Growth	1 in 1000
2. Chloroform soluble fraction.	<i>S. aureus</i>	"	"
3. Acetone soluble fraction.	<i>B. coli</i>	No growth	"
4. Ether soluble fraction.	<i>S. aureus</i>	"	"
5. Petroleum ether soluble fraction.	<i>B. coli</i>	Growth	"
	<i>S. aureus</i>	No growth	"
	<i>B. coli</i>	"	"
	<i>S. aureus</i>	Growth	"
		"	"

The protein obtained from garlic by isoelectric precipitation was separated, washed and brought into solution; the pH of the solution was adjusted to 7. The solution was diluted to a concentration of 1 in 1000 and candle filtered. The filtrate was tested by serial dilution method against *B. coli* and *Staph. aureus* and was found to be inactive.

With the above preliminary observations the following method was developed for the preparation of the active principle. It was found to yield a sufficiently active product.

1 kg. of garlic cloves after removal of the root hairs and dry skin is cooled to 0°C in ice box for 2-3 hours. The cloves are then thoroughly crushed in a previously cooled mincer and at once laked with 2 litres of cold (5°C) distilled water. The whole extract is kept overnight in a frigidaire. The solution is filtered by means of a lint cloth and the juice is pressed out of the cells. The aqueous extract is slightly turbid. This is adjusted to pH 3.8 with 10% sulphuric acid and the proteins are precipitated. The supernatant fluid is centrifuged and the centrifugate kept in frigidaire. The proteins are washed with 200 cc. portion of distilled water and again centrifuged. The wash is added to the original supernate. The pH of the solution is raised to 6. The total aqueous extract (2.5 litres) is concentrated under reduced pressure and low temperature to half its volume. The concentrated solution is then extracted twice with 200 cc. portions of ethyl ether and the ethereal layer containing the active substance is kept in the frigidaire for further work. It

is treated with anhydrous  $\text{Na}_2\text{SO}_4$  to remove moisture. The ether is removed at a reduced pressure of 15-20 mm. The residue left after removal of ether is treated with 250 cc. of cold water whereby an emulsion is obtained. The emulsion is treated with 10 cc. of n-hexane to remove the yellow pigments and other hexane soluble substances. The aqueous extract is separated and again shaken twice with 50 cc. portions of ether. The ether is again concentrated at reduced pressure (10-15 mm.). The residue is a colourless oily liquid which solidifies on keeping at room temperature. The oily substance is found to be the active antibacterial substance of garlic. The yield is 0.5—1 g. per kg. The substance is dissolved in sterile redistilled water, centrifuged and the clear centrifugate is kept. The solution was diluted to contain 0.1 mg./cc. and kept in frigidaire for anti-bacterial investigation.

The test of antibacterial activity was carried out by following the serial dilution method. The broth used containing 1% glucose, 1% peptone, 0.5% sodium chloride and meat extract, adjusted to pH 7. 5 cc. of sterile broth was pipetted aseptically into sterile tubes. One loopful of bacterial emulsion of test organism in normal saline (1000 million/cc.) was the inoculum used in the 5 cc. broth. Phytoncide extract was added to the culture tubes to maintain concentration of 1 in 50,000, 1 in 100,000 and 1 in 150,000. A control tube containing the organism without phytoncide extract was run in each case. The culture tubes were incubated for 18 hours at  $37^\circ\text{C}$ . The growth was observed after the incubation period, the end point of inhibition was sharp, the effective concentration showing no growth, the next in the series showing good growth. The concentration showing no growth was tested for bactericidal or bacteriostatic action by removing one loopful of culture solution into sterile 5 cc. glucose peptone meat extract broth. Re-inoculation showed no growth showing bactericidal nature of the garlic phytoncide. Other workers claimed bacteriostatic nature of the garlic phytoncide, but our results indicate bactericidal nature. Table II summarises the results obtained.

TABLE II

Organism studied.	Concentrations of garlic phytoncide in broth		
	1 in 50,000	1 in 100,000	1 in 150,000
<i>Staphylococcus aureus</i>	No growth	No growth	Growth
<i>Streptococcus hemolyticus</i>	"	"	"
<i>B. coli</i>	"	"	"
<i>B. typhosum</i>	"	"	"
<i>V. cholerae</i>	"	"	"
<i>B. paratyphosum A</i>	"	"	"
<i>B. paratyphosum B</i>	"	"	"
<i>B. dysenteriae shiga</i>	"	"	"
<i>B. dysenteriae flexner</i>	"	"	"
<i>B. proteus</i>	"	"	"

The active substance is volatile and irritating to the skin and the odour is characteristic of garlic. It is soluble in alcohol, ether and chloroform. It has been a common observation during the process of extraction that the active substance when kept at room temperature in contact with air, solidifies into a resinous mass.

within 2 to 3 days. In such cases it is very difficult to get it dissolved in water and if solution at all is possible, it shows marked diminution in activity. This might be due to some inter molecular rearrangements or some polymerization resulting in the formation of a substance which is not antibacterially very active. It is interesting to note that the active substance which keeps fairly well in the garlic cells before extraction loses its activity so quickly after its isolation in pure form.

### DISCUSSION

The name 'Phytoncide' has been used by the authors to indicate the bactericidals in plants. The volatile emanations undoubtedly possess anti-bacterial activity. The substance isolated by the authors is volatile. But from the experimental results they cannot support the view of Toroptsev and Filatova (2) that the anti-bacterial activity is present only in the emanations and a short exposure in air deprives the crushed cells of their active substance. The active substance has not been found to be so highly unstable.

Regarding our observations on the activity of the different fractions of garlic obtained by solvent extraction, results of which appear in Table I, it might appear paradoxical that though the active substance is miscible with alcohol still it is found that the alcohol soluble fraction does not show any activity. Moreover, the active substance has been found by Cavallito and Bailey (6) and Rao *et al* (8) to be present in the alcohol soluble fraction of garlic. But we believe that the alcohol soluble fraction over and above containing the phytoncide contains some other substances which, as if, poison or inhibit the activity of the phytoncide. For, though Cavallito and co-workers have started with the alcohol soluble fraction for the isolation of the anti-bacterial substance, they have ultimately used the alcohol soluble substance volatilizing at a reduced pressure of 10-15 mm., whereby it is freed from interfering inhibitory substances. Rao *et al* (8) have also worked with the chloroform soluble fraction obtained from the alcoholic extract of garlic. Our extraction process eliminated the use of alcohol and since the active substance is soluble in waer at low concentrations we have availed this property of the active substance in isolating it.

The substance isolated by following our method is active at a maximum dilution of 1 in 100,000 which shows it to be almost as active as allicin, extracted by Cavallito, which is anti-bacterial at a maximum dilution of 1 in 125,000. The substance isolated by us appears to be more bactericidal than bacteriostatic while the effect of allicin is more bacteriostatic than bactericidal as is claimed by Cavallito *et al* (6) and Rao *et al* (8), but it cannot be called strictly bactericidal or bacteriostatic unless the mechanism of its antibacterial activity is thoroughly studied. Though Cavallito *et al* (9) have claimed the active substance in garlic to be a sulphhydryl-inhibiting substance in which case it is supposed to act by depriving bacterial cells of their metabolic requirement of substances containing SH groups, it is not unlikely that the active substance may prove an anti-enzyme, if we are allowed to say so, affecting the activity of the enzyme or enzymes connected with some essential biologic cycles of the bacteria.

## SUMMARY

1. The volatile emanation obtained from garlic is of lethal effect to *staphylococcus aureus* and *streptococcus hæmolyticus*.
2. The active substance is present in ether and chloroform extractives.
3. A method of extraction has been described and the active substance is bactericidal at a dilution of 1 in 100,000 against both gram-positive and gram-negative organisms.

Further work on the protistocidal and fungicidal properties of the garlic phyto-cide and also of the therapeutic significance in experimental diseases is in progress.

Our thanks are due to Dr. B. C. Guha, D.Sc., F.N.I. for helpful suggestions and for a grant to the Indian Research Fund Association for carrying on this investigation.

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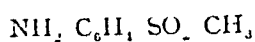
ON THE ANTIBACTERIAL ACTIVITY OF SULPHONE DRUGS

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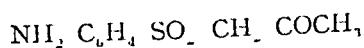
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Diasone, promin, sulphetrone,—all derivatives of p,p'-diamino diphenyl sulphones—, are now being used in the treatment of leprosy with certain advantage. The parent compound is, however, extremely toxic and this observation at the early stage of Sulpha therapy (1) most probably has not led to any systematic

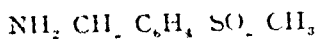
investigation on this class of compounds. Chance observation even indicates the efficacy of sulphone against gas gangrene (Evans *et al* 2). It is now known that Sulphanilamide derivatives are generally acting through the 'same' mechanism, and their different activity *in vitro* being explained by differences in degree of adsorption to the bacteria (3). A possibility, however, always remains that the activity may be enhanced by the introduction of a substituent into the sulphonamide molecule (Julius and Salomen, 4). There are compounds like p-aminomethyl benzene sulphonamide (Marfanil), and p-amino benzene sulphon-(3, 5-dibromo phenyl) amide which act through different mechanisms (Jensen *et al*, 5) and Goetchins and Lawrence (6). It has been noticed (Basu, Sen Gupta and Sikdar, 7) that p-amino methyl sulphone (I) previously studied by Buttle *et al* (8), is an extremely weak acid with a pka value of 11.0 and exerts a very low *in vitro* activity against bacillary dysentery organism (Flexner, Y). It is being found to possess no special advantage over pure sulphanilamide. The substitution of the hydrogen atom of the methyl group by one electrone attracting radical,  $-\text{CO}\cdot\text{CH}_3$ , (Bell and Roblin, 9) afforded p-amino phenyl acetonyl sulphone (II) with an increase in the acid dissociation constant (Basu *et al*, 7). Similarly the replacement of the p-amino grouping by p-amino-methyl grouping afforded a compound (III). The bacteriostatic activity of all these compounds (10) along with (IV), (V) and (VI) were studied against certain gram-positive and gram-negative organisms, but none of the p-amino phenyl sulphone derivatives showed any enhanced activity. The sulphone analogue of marfanil, p-amino methyl phenyl methyl sulphone is the only compound that requires further study.



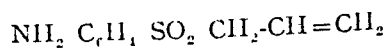
(I)



(II)



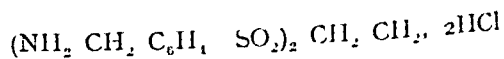
(III)



(IV)



(V)



(VI)

**Bacteriostatic Activity:**—For these stock laboratory strains of different organisms as mentioned in the Table below were cultured for a period of 18-24 hours in a medium made from papain-digest glucose phosphate meat broth, and the fresh cultures were incubated with the different products in the same medium at 37° for 72 hours. The Table shows the milligram of the drug per 100 c.c. of culture medium that was necessary for inhibiting the growth of different organisms under the conditions of the experiments described.

TABLE I

Number of organisms in each case		...	1000 per 5 cc.				
Period of Incubation ..		...	72 hours				
Figures indicate milligram of the drug per 100 cc. of the medium.							
Organisms	Products.						
	I	II	III	IV	V	VI	Marfanil
B. dysentery (Shiga)	—	20	2	100*	100*	100*	100
Strep. hæmo.	20	100*	1	100*	100*	100*	2
Stap. aur.	10	100*	2	100*	100*	100*	10
Pneumo. I	20	100*	1	100*	100*	100*	2
B. coli	—	100*	50	100*	100*	100*	100
B. typhosum	--	100*	50	100*	100*	100*	50

\* The figures marked with asterisks showed no bacteriostasis.

From the Table it is evident that these compounds are not active and there is no appreciable difference in their *in vitro* activity. The p-amino methyl benzene sulphonamide (marfanil) as well as its sulphone analogue (III) is, however, somewhat active against the gram-positive organisms. If chemo therapeutic activity be conceived in terms of interference at some particular stage in the progression of metabolic reactions involved in the parasites (Fildes, 11), no relationship is being established between the chemical structure and bacteriostatic activity in this class of sulphones. Various hypotheses are being put forward for the mode of action of sulphonamide drugs but seldom attention is being drawn to other similar type of compounds which have also been found to be potent in cases where sulphonamide drugs have failed. To such group belongs marfanil, or, sulphone whose chemistry and mode of action need further investigations.

### CONCLUSION

A study on the bacteriostatic activity of certain sulphones against a low number of organisms both gram-positive and gram-negative, does not reveal any relationship between chemical constitution and bacteriostatical activity in this class of compounds. The characteristics of marfanil and p-amino methyl phenyl methyl sulphone, however, point to a systematic study on the mode of action of this class of compounds so that organic chemists may obtain beneficial results for mankind by more synthetic work in this domain.



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PLASMA COPPER CONTENT OF HEALTHY INDIAN ADULTS

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The occurrence of copper in the blood of man and various animals have been known for a long time. With the discovery of the essential nature of copper for haemoglobin formation (1), attempts have been made during the last few years to study systematically, the copper content of blood of man in normal and pathological conditions. Several observations are recorded about the changes of the copper

content of the blood in various conditions of anaemia. Schultze, Elvehjem and Hart (2), made a detailed investigation on the changes in the copper content of the blood of animals, suffering from nutritional anaemia under well controlled conditions and the conclusions from these investigations are of interest, as it is generally accepted that deficiency of copper causes nutritional anaemia. They found that in pigs suffering from nutritional anaemia due to iron and copper deficiency, the copper content of blood falls to extremely low levels.

Several investigators have reported that copper content of blood is increased in different types of anaemia other than nutritional anaemia and also in pregnancy (3).

In view of these observations, it is of interest to determine the copper content of blood of healthy Indian adults.

### EXPERIMENTAL

Sodium diethyl dithiocarbamate has been found to be a satisfactory and sensitive reagent for the determination of copper. McFarlane (4) found that the coloured copper complex could be rapidly and quantitatively extracted from aqueous solution by amyl alcohol and that the colour was intensified in the organic solvent. Iron interferes by producing a brown colour, which can be prevented by the addition of sodium pyrophosphate.

Locke, Main and Rosbash (5) prepared a protein free filtrate of serum by precipitating the proteins in the cold with tri-chloroacetic acid and determined the copper content of the filtrate with the carbamate reagent. Tompsett (6) demonstrated that the whole of the serum copper is present in such a filtrate and is present in such a way that it can be determined by carbamate. Using such a procedure, he obtained excellent recoveries and the results compared closely with those obtained by ashing. Yoshikawa, Hahn and Bale (7) using radioactive copper, found that nearly all of the copper in plasma is bound in some manner to protein and only about two-third is split off with tri-chloroacetic acid in the cold. Cartwright, Jones and Wintrobe (8), have shown that three warm extractions of the tri-chloroacetic acid precipitate removes about 97% of copper. In their opinion, the method not only gives excellent recoveries of added materials but offers results which are consistently reproducible  $\pm 10\%$ . In the present investigation, this method has been followed but the golden yellow colour developed with the carbamate reagent has been extracted with amyl alcohol for matching in Evelyn Photoelectric Colorimeter, using Filter No. 440. In the method proposed by Cartwright *et al* (8), the coloured aqueous solutions were read directly in the Photoelectric Colorimeter without extracting the colour with amyl alcohol. This procedure was also tried, but as the coloured solution was rather hazy, it was decided to extract with amyl alcohol, in which the colour was intensified. Glass distilled water was used throughout the investigation.

Copper content of plasma of 25 healthy adults males and 10 healthy females have been estimated. The results are presented in Table I

TABLE I

Subject	Range $\gamma$ /cent	Average $\gamma$ /cent		Standard Deviation
Males	98-182	142	11.2	28.3
Females	121-187	155.3	18	27.8

The frequency distribution is depicted in Fig. I.

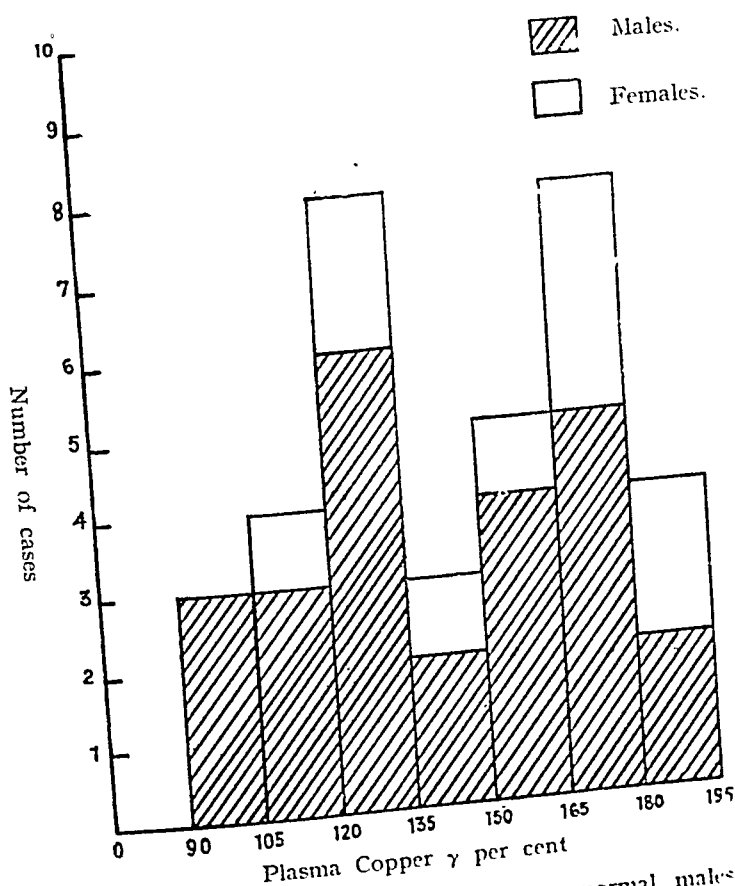


FIG. I Plasma Copper Content of 25 normal males and normal females.

### DISCUSSION

The average for males is 142.17 per cent. and that for females is 155.3 per cent. This higher average value for females correspond with the same finding by Cartwright *et al* (8). The results of the present investigation and that obtained by various investigators in different countries have been summarised in Table II.

was estimated by the usual chemical method with 2:6-dichlorophenol-indophenol (Harris *et al.*, 2). The dye was previously standardised with known amount of vitamin C. The amount of vitamin C excreted daily was thus obtained. The results are given in tables I, II and III.

TABLE I

*The urinary excretion of vitamin C by the normal rats. The results are expressed in mg. per 24 hours period.*

No. of rats.	Vitamin C values on										
	1st	2nd	3rd	6th	7th	8th	9th	10th	12th	13th	14th day.
1	0.16	0.10	0.69	0.43	0.38	0.98	0.57	0.65	0.14	0.83	0.23
2	0.13	0.10	0.10	0.10	0.10	0.20	0.34	0.29	0.20	0.54	0.47
3	0.97	1.20	1.50	0.79	1.57	0.94	1.60	1.20	1.40	1.50	1.70
4	0.30	1.10	1.05	0.68	0.40	—	1.30	0.95	0.55	0.57	0.79
5	0.38	1.30	1.40	1.08	1.50	1.80	1.74	1.67	3.42	3.04	3.00
6	—	—	—	—	—	0.94	0.87	0.26	0.76	0.30	0.15
7	0.11	0.34	0.30	0.20	0.15	0.38	0.33	0.33	0.44	0.38	0.21
8	1.10	1.30	1.90	1.60	0.95	1.80	1.10	1.60	1.70	2.20	2.20

TABLE II

*The urinary excretion of vitamin C by rats, treated with cobra venom (1/300 mg./100 g. bodyweight/day) for a period of two weeks. The values are expressed in mg. per 24 hours period.*

No. of rats.	Vitamin C values on										
	1st	2nd	3rd	6th	7th	8th	9th	10th	12th	13th	14th day.
1	0.57	0.14	1.90	3.15	1.58	1.97	1.97	1.93	2.00	1.85	2.00
2	0.16	0.10	0.34	0.20	0.15	0.28	0.22	0.17	0.15	0.42	0.18
3	0.97	0.48	1.30	0.76	0.76	0.63	0.48	0.95	0.42	0.56	0.47
4	0.35	0.25	0.43	0.63	0.57	0.94	0.56	0.42	0.29	1.05	0.40
5	0.11	0.29	0.42	0.23	0.20	0.20	0.20	0.20	0.13	0.13	0.36
6	—	1.52	1.60	0.90	1.70	1.77	—	1.64	1.55	1.60	0.93
7	0.11	0.51	0.78	0.15	0.20	0.76	0.76	0.66	0.60	0.50	0.14
8	—	0.38	0.41	0.30	0.20	0.34	0.29	0.30	0.29	0.34	0.13

TABLE III

*The urinary excretion of vitamin C by rats treated with cobra venom (1/150 mg./100 g. bodyweight/day) for a period of one week. The values are expressed in mg. per 24 hours period.*

No. of rats.	Vitamin C values on						
	1st	2nd	3rd	4th	5th	6th	7th day.
1	1.20	1.06	1.10	1.00	0.93	1.18	1.00
2	0.73	1.00	0.98	0.76	0.69	1.10	1.00
3	0.68	0.60	0.70	0.67	0.51	0.61	0.71
4	0.44	0.62	0.50	0.48	0.54	0.71	0.27
5	0.25	0.25	0.26	0.42	0.66	0.65	0.44
6	0.10	0.10	0.20	0.24	0.40	0.38	0.39
7	0.10	0.10	0.20	0.20	0.20	0.10	0.20
8	0.10	0.10	0.10	0.48	0.20	0.64	0.20
9	0.10	0.10	0.20	0.20	0.20	0.20	0.10

The reserve of vitamin C in different tissues such as liver, kidney, brain, and heart of the rats, normal or treated with venom was determined. Tissue was taken from freshly killed animals, freed from blood and adhering membranes. Weighed amount was thoroughly ground with sea sand (Merck) in 2 cc. portion of 20% trichloroacetic acid, and centrifuged. The clear extract made up to a definite volume, was titrated in the usual manner with 2:6-dichlorophenol-indophenol to determine its vitamin C content. The dye was previously standardised with known amount of vitamin C. The results as obtained in the various cases are given in the table IV.

TABLE IV

*The amount of vitamin C in the brain, liver, kidney, and heart of rats normal and treated with venom, (1/150 mg./100 g./day and 1/300 mg./100 g./day). The values are expressed in mg. of Vitamin C.*

Source of rats.	No. of rats.	mg. vitamin C per g. of fresh			
		Liver	Kidney	Brain	Heart
I Normal rats.	1	0.2666	0.1904	0.4444	0.0599
	2	0.2666	0.1600	0.4444	0.0760
	3	0.2628	0.1754	0.4222	0.0658
	4	0.2600	0.2380	0.6289	0.0700
	5	0.2222	0.1739	0.4933	0.0800
	6	0.2354	0.3000	0.5000	0.0940
	7	0.3070	0.2807	0.5000	0.1212
	8	0.3300	0.2152	0.4444	0.0914
	Average	0.2694	0.2167	0.4847	0.0823
II Rats treated with 1/300 mg. venom/100 g./day for two weeks.	1	0.2857	0.2500	0.5000	0.0842
	2	0.1904	0.1666	0.4000	0.0727
	3	0.2000	0.1904	0.4800	0.0941
	4	0.1818	0.1666	0.4000	0.0701
	5	0.1429	0.1851	0.4000	0.1000
	6	0.2354	0.2666	0.5290	0.0563
	7	0.1333	0.1428	0.3666	0.0768
	Average	0.1956	0.1954	0.4304	0.0792
Rats treated with cobra venom (1/150 mg. per 100 g. bodyweight per day) for one week.	1	0.1256	0.1280	0.2588	0.0600
	2	0.2354	0.1600	0.2750	0.0700
	3	0.2010	0.1804	0.2855	0.0740
	4	0.1660	0.1355	0.2000	0.0730
	5	0.2000	0.2400	0.2600	0.0740
	6	0.2000	0.2070	0.3150	0.0780
	7	0.1660	0.1660	0.2500	0.0500
	8	0.1430	0.2000	0.2041	0.0700
	9	0.2000	0.2000	0.2354	0.0700
	Average	0.2042	0.1806	0.2748	0.0700

## DISCUSSION

The effect of cobra venom, so far as the biosynthesis of vitamin C by the rat is concerned, is negligible. From tables I, II, and III it appears that the urinary excretion of vitamin C is unaffected by treating the animals with sub-lethal-doses of Cobra venom.

The vitamin C reserve in different tissues, however, seems to be affected by cobra venom. The figures in table IV show that rats treated with venom show some depletion in the vitamin C content of the tissues. The percentages of depletion in the different tissues are given below:

TABLE V

Percentage of depletion of vitamin C

	1/300 mg. per 100 g. body- weight.	1/150 mg. per 100 g. body- weight.
Brain	12.22	45.04
Kidney	10.11	16.92
Liver	27.62	26.00
Heart	3.00	17.50
Tissue.		

Here it is found that of all tissues affected, the brain shows the highest depletion for the higher dose of venom (45.04%). Liver is affected to almost the same extent in the two doses. Kidney and heart are affected to a greater extent for the higher dose.

Hence it is found that so far as synthesis of vitamin C is concerned, the urinary excretion or tissue reserve, the effect of cobra venom injection is not allied to that of the narcotics.

## ACKNOWLEDGMENT

The author wishes to express his sincere thanks to Dr. J. C. Ray M.D., F.N.I., Director, Indian Institute for Medical Research, for giving him all facilities in conducting the investigation. He is indebted to Dr. S. C. Roy, D.Sc. of University College of Science, for valuable suggestions in carrying out the work.

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**FURTHER STUDIES ON COMPLEMENT-FIXATION REACTION IN  
KALA-AZAR WITH A SPECIFIC ANTIGEN AS AN  
AID TO DIAGNOSIS**

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(Received for publication, May 25, 1949)

In continuation of our preliminary studies on the complement-fixation reaction in kala-azar with a specific antigen prepared from *Leishmania donovani* (1), further



work has been done with a view to improve the quality of the antigen and technique of the test and also to verify the dependability of this reaction. In order to achieve these, various methods for preparation of antigen have been tried, the technique of the complement-fixation reaction in kala-azar has been modified a little from that of our previous work and the test has been carried out in fairly large number of cases.

### PREPARATION OF COMPLEMENT-FIXING ANTIGENS

Several recent strains of *Leishmania donovani* isolated from spleen or sternal puncture fluid of kala-azar patients were primarily cultivated in N.N.N. media and subsequently subcultured in Ray's media. When sufficient growth was available, different methods for preparation of antigens were attempted.

(I) *By shaking method*: An emulsion of 72 hours' growth of the flagellated culture of *Leishmania donovani* was made with distilled water, the proportion of parasites to solvent being 1 in 50, i.e. one gramme growth without admixture of the media was added to 49 cc. of distilled water. This proportion almost tallied with 80 million organisms per cubic centimeter. 0.5% phenol was added as preservative. These were kept together with some sterile sand in a sterile glass stoppered bottle in an incubator at 37°C for 25 days. The bottle was taken out every day and shaken for two hours in a shaking machine having 120 oscillations per minute. Finally, the emulsion was centrifuged and the supernatant fluid was kept in refrigerator as antigen. This antigen was prepared exactly in the same manner as the one described in our previous contribution except that 0.5% phenol was added to distilled water instead of 0.25% phenol, in order to ensure better sterility. As before this antigen was found to be highly potent when tested for complement-fixation reaction against anti-kala-azar rabbit's immune serum. The titre in which a positive fixation was obtained varied from 1 in 4000 to 1 in 6000. The potency of the antigen remained unaltered from 10 months to 1½ year. This antigen was employed for carrying out the test in routine work as it was found to be the best.

Antigen prepared with normal saline according to the same technique was found to be less potent.

(II) *By trituration method*: Different antigens were prepared by triturating flagellated form of *Leishmania donovani* with sterile fine sand till all the parasites were broken into pieces as seen under the microscope. Extractions were then made with different solvents, such as 0.5% phenol in distilled water, 0.5% phenol in normal saline, ether, alcohol and acetone. The proportion of parasites to the extracting fluid was 1 in 50 in every case. The emulsions were centrifuged at high speed and the supernatant fluids were pipetted off and kept in the refrigerator as antigens.

Acetone extract gave a greenish discolouration when used and the alcohol and ether extracts were very weak in antigenic potency and thus they were rejected.

Antigen prepared with normal saline did not prove as good as that prepared with distilled water. The distilled water extract containing phenol as preservative was found to be quite potent giving a complement-fixation titre of 1 in 2000 to 1 in 4000 with immune rabbit's serum. This antigen took about a month's time to mature before it could fix antisera to such high titres. But after a period of 4 to 5 months the potency began to decline with settling of sediment. This was the only antigen which could be compared favourably with the antigen prepared from distilled water by shaking method.

(III) *By freezing and thawing*: Another variety of antigen was tried in which the parasites were ruptured by alternate freezing and thawing in distilled water with 0.5% phenol. Though the antigen showed very good result in its potency, yet it had to be rejected because of the presence of haziness in all the tubes containing the antigen during actual test.

#### MODIFICATIONS IN THE TECHNIQUE OF THE COMPLEMENT-FIXATION REACTION

(a) *Titration of antigen for its anti-complementary activity*: It was found that with freshly prepared antigens, the same results were obtained irrespective of the fact whether the antigen dilutions used were titrated against 1 or 2 m.h.d. of complement, though, 1 m.h.d. of complement was always used for the actual titration. But with aging of antigen, it was found that better results could be obtained when antigen dilutions, titrated against 1 m.h.d. of complement, were used. So in carrying out the test proper antigen dilution, titrated against 1 m.h.d. of complement, was employed.

(b) *Complement-fixing power of the antigen*: For determination fixing power of the antigen, high titre rabbits' immune sera, prepared against *Leishmania donovani*, were used. Method of preparation of the immune serum was same as mentioned in the previous communication (1). The technique employed to ascertain the complement-fixing power of antigens was exactly similar to that carried out during the test proper. Usually the complement-fixing titre of a potent antigen varies from 1 in 2000 to 1 in 6000 or more.

To ascertain the strength of the antibodies in the immune serum, agglutination reaction was also carried out with flagellate form of *Leishmania donovani* (2). When agglutinating titre was 10,000 or above, the antigen was tested for its complement-fixing power with the immune serum. To detect any deterioration in potency of the used or old antigen, complement-fixation reaction was done at varying intervals with immune serum which kept for fairly long time.

(c) *Test Proper*: The technique of the test proper was more or less the same except the following alterations:—

## REMARKS

The value of the complement-fixation reaction in kala-azar with a  $S_{12}$  antigen lies in specific and early diagnosis of kala-azar. Since the test is a  $S_{12}$  antigen-antibody reaction, positiveness of the reaction depends only upon antibody produced during the course of illness. Besides, the test is of value in guidance of treatment as complement-fixing antibody in the blood begins to appear with the attainment of cure.

## ACKNOWLEDGEMENT

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NATURE AND SPECIFICITY OF URINARY PROTEOSE AS  
PART I. THERAPEUTIC TRIAL OF URINARY PROTEOSE AS  
HYPOSENSITISING AGENT IN ALLERGIC CASES

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Distinction between an endogenous allergy from an exogenous condition becomes a difficult problem, unless the patient narrates his particular history. Moreover two conditions may co-exist and separate identification is essential for handling of the state scientifically. Diagnosis of infection in host has been, to a great extent, obviated by various serological methods based on immunological reactions; allergy being a pervert immunity response, such immunological reactions may be utilized in the diagnosis of allergic state. In fact it has been, to some extent, practised in exogenous varieties of allergy, and it is hoped that in future with modified techniques, compatible with the complicated process of allergic state, identification of endogenous allergic condition is mainly due to non-availability of the antigen which may allergy may be possible. The difficulty in such a technique for detection of endogenous allergic condition is mainly due to non-availability of the antigen which may be incriminated to be the factor responsible for this pathological entity, or in short the nature and form of endogenous allergen has not yet been established. Urinary proteose of Oriol is regarded to be an allergen responsible for endogenous allergy, but no scientific attempt has yet been made to prove its guilt. So long work on urinary proteose were mainly clinical in nature. No study on its allergenic nature and its relation with the organism directly responsible for the condition has been made. This communication deals with such an attempt by the authors and opinions expressed therein are based on their preliminary observations on a limited number of cases.

I. THERAPEUTIC TRIAL OF URINARY PROTEOSE AS HYPOSENSITIZING AGENT  
IN ALLERGIC CASES

Auto-uro-proteose therapy is one of the much discussed subjects in the science of allergy. Medical literature has recorded a huge controversy among equally eminent workers on the efficacy of urinary proteose in the treatment of allergic conditions. Credit must go to Oriol and Barber (1,2,3,4) who during systematic study of allergic cases first detected and isolated a proteicous substance from the urine of allergic patients particularly during recurrent crises of the condition. They noted that when acidified urine is shaken with ether it forms a white turbid emulsion. Addition of alcohol to this gel will precipitate a white substance which collects at the top.

They brought the precipitate to solution, prepared their stock solution of 1:1000 and made the following important observations:

1. That urinary proteose is excreted in much greater amount in allergic patients particularly during crises.
2. That urinary proteose is excreted by normal person but much less in quantity.
3. That allergic patients are sensitive to their own proteose while normal persons are not so nor do they show reaction to proteose from other allergic patients.
4. That proteose therapy produces encouraging results.

These observations have been scrutinised by various workers and their opinions are reviewed below. Conybeare (5) reported that protein-sensitive cases showed high degree of sensitivity to auto-proteose while non-protein-sensitive cases exhibited low degree of reactions. Non-allergic subjects showed no reaction at all. Darley and Whitehead (6) noted in their series positive reactions during active phase of the disease (asthma) and negative reactions during asymptomatic period. Positive reactions were observed in arthritis and serum sickness, while negative in eczema, urticaria and erythema multiforme. Burgess (7) obtained positive skin reaction to auto-proteose in 69 per cent of allergic patients and 16 per cent of normal cases, while Yeoman *et al* (8) observed 50 per cent positive reactions in rheumatoid arthritis. Bruce Pearson (9) in his series recorded high degree of positive reactions in protein sensitive asthmatics, while non-protein sensitive cases showed lower sensitivity. No specificity was, however, claimed. Eichenlaub (10) got positive skin reactions in three of his skin cases. Freeman (11), on the other hand, denied any specific property of urinary proteose from allergic cases, reactions in his series being totally non-specific and differing in no way from normal cases. Murray *et al* (12) supported Freeman's observation in his own series of 25 allergic cases. Steel (13) and Van Leeuwen (14) reported not only negative response to auto-proteose, but that cases with negative response to auto-proteose showed definite sensitiveness to hetero-proteose. Minchin (15) in his series of ten cases of epilepsy could not elicit any response. Aldred-Brown and Munro (16) examined 50 cases of rheumatoid arthritis with no skin reactions. Cornia (17) has suggested the skin reactions due to local irritations while Cornbleet and Kaplan (18) to salts and other irritants in proteose solutions. Libman and Bigland (19) did not observe any appreciable skin-sensitivity in allergic patients to auto-proteose.

12 and 6 cases of hay fever (21). Watson's (22) cases of hay fever responded to proteose therapy. In rheumatoid arthritis Oriel and Barber (3) reported good effect on conditions of joints though the associated skin troubles did not show any change. Yeoman *et al* (8) supported their observations and got good results in rheumatoid arthritis. Andred-Brown and Munro (16) had no appreciable effect. Templeton (23), Cornbleet and Kaplan (18) got unsatisfactory results in skin cases, so also Meranze *et al* (24) in asthmas. According to Libman and Sigland (19) proteose therapy is no better than peptone treatment. Any specificity of the former, if present, is outweighed by the advantages of the latter. But they could observe encouraging results in urticaria. Such a dispute over a clinical opinion among equally eminent workers undoubtedly creates an anomalous position. Urbach (25), however, has tried to settle this controversy by suggesting auto-uro-proteose therapy in endogenous allergic cases only. In this series of cases we have tried to utilize uro-proteose-therapy in endogenous variety of allergy only by the method of hyposensitisation.

#### SKIN SENSITIVITY TO AUTOPROTEOSE

In the present series, 27 cases of allergic diseases have been treated, of which 18 were asthma, 5 dermatitis, 1 rhinitis, 1 urticaria, 1 psoriasis and 1 rheumatoid arthritis. The method of extraction of urinary proteose has been mainly based on Oriel's (20) modified technique. Their method of extraction as advocated by Urbach (25), which entails vigorous treatment of such a delicate substance with alcohol and subsequent drying in incubator was avoided, as according to Oriel, drying or even standing the substance for sometime may denaturise it vitiating thereby its cutaneous reactivity and therapeutic effect. The method of extraction, as followed in this piece of work, is detailed below.

24 hours' sample of urine from an allergic patient during symptomatic crisis was collected in sterile bottles containing toluene as preservative and kept in ice-pack during collection. The cooled urine on the next day was passed through a Seitz's filter and 400 cc. of this filtered urine was then acidified with 25% sulphuric acid till it was just acidic to freshly prepared congo-red paper. Acidified urine was then shaken with 100 cc. of ether and allowed to stand in ice-chamber for half-an-hour. The layer of gel at the top was retained and treated with ice-cold 95 per cent alcohol and the flaky precipitate thus formed was settled in cold chamber overnight. The precipitate at the bottom was collected by centrifugalisation in sterile tubes, washed in distilled water and recentrifuged. It was then readily treated with N/10 NaOH till it went fairly to solution, and the whole thing was diluted to 10 cc. volume with Evan's buffer solution. This was the stock solution of urinary proteose in 1/1000 strength. This qualitative expression of Oriel was preferred to the quantitative strength, by weight, of Urbach so as to avoid drying the matter in the process which might denaturise it.

In the clinical trials with urinary proteose solutions, as a rule, a preliminary skin test on all patients by intracutaneous methods was made with different dilutions, 1/10,000, 1/20,000, 1/50,000, 1/75,000 and 1/100,000. A control test with Evan's solution was always carried out to rule out any false positive reaction.

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By positive reaction it is meant that a definite blanched wheal with erythematous zone around it (with or without pseudopodia) would appear within half to one hour.

*Method:*—Skin of the anterior aspect of the fore-arm or back was selected for skin testing. 0.1 cc. of proteose solution of highest dilution (1/100,000) was injected intracutaneously at one site; if no reaction appeared within 15 to 20 minutes, 0.1 cc. of next lower dilution of proteose solution, as specified above, was used, and so with other dilutions till the last one of 1/10,000 was tested. 0.1 cc. of Evan's solution was injected to serve as control. Two sites of injection were so planned as to lie two inches apart laterally and three inches vertically in order to eliminate overlapping of reactions. Positive skin reactions usually started to appear within 15 to 20 minutes. Sensitivity of skin could be enhanced by previous preparations of the sites with 0.1 cc. of patient's serum intracutaneously 24 to 48 hours before the test with proteose solution. In one of our cases, however, serum sensitized sites showed no reaction to proteose but non-sensitized sites in the other arm presented good response. This might be due to absence of skin sensitizing antibodies or presence of antigen blocking antibodies in the serum of patient. The antigen blocking antibodies blocked the proteose, the suspected antigen, before it could combine with skin sensitizing antibodies at the sites.

In this series no cases showed negative reaction to auto-proteose. Cross reaction with hetero-proteose was not examined in each case. In a very few so tested hetero-proteose did not elicit any reaction though the patients were sensitive to their own proteose solutions. The reactions persisted for varying periods, from 2 to 18 hours, but what was reckoned as a more significant fact was a relation of symptom—complex to skin testings. Some patients experienced acute aggravation while some other complete amelioration of the symptoms.

*A case of neurodermatitis, female, 32 years.* Skin test was positive even with 1/100,000 dilution. Local reaction after 24 hours was nil but skin eruptions around the mouth completely faded and a neuralgic throbbing pain in the ear was much relieved.

*An asthmatic, male, 40 years.* Skin test was positive with 1/50,000 dilution. Patient had no lapse in asthmatic fits even with adrenergic drug-administration before skin testing, but he was practically free from symptoms 24 hours after the test.

*A patient of urticaria, male, 55 years.* Skin test was positive with 1/20,000 dilution. He complained on the next morning of acute exacerbation of itching all over the body even inside the mouth and urethra.

*A case of cheilopompholyx, male, 20 years.* Skin test was positive with 1/75,000 dilution. The patient experienced exacerbative symptoms.

*A case of psoriasis, female, 18 years.* Skin test was positive with 1/75,000 dilution. Lesions were more itchy in the night after skin testing.

*A case of rheumatoid arthritis, male, 30 years.* Skin test positive with 1/50,000 dilution. The patient did not notice any change.

Hyposensitisation with proteose solution has been given a trial in this series. The course of treatment was fairly prolonged and consisted of intracutaneous or subcutaneous injections at suitable gaps. The starting dose was based on the skin-sensitivity of the case and like Oriel's method commenced from a strength ten times diluted to that which gave a definite skin reaction in the subject. The quantity was 0.1 cc. which was gradually increased by 0.1 cc. depending on the reaction of the dose administered. After 1 cc. the next lower dilution was used and the doses were worked up in the stated manner depending on the reaction of the patient. After 5 or 6 injections therapeutic effect was usually noticed though it might appear later than usual expectations. Some of the cases of the series resisted any apparent improvements. Total cure was seldom experienced (for one year). Relapses were often met with. Reinstitution of auto-proteose therapy sometimes improved the condition but the progress seemed to be definitely slow. It failed totally in some of the cases. Results of therapy and skin testing are denoted in the following chart.

*Result of Auto-proteose therapy.*

Disease.	No. of cases.	Positive skin test.	Therapeutic results			
			Total	75%	50%	Nil
Asthma	18	18				
Dermatitis	5	5	5			4
Urticaria	1	1	3	8	1	1
Rhinitis	1	1	—	1	—	—
Psoriasis	1	1	—	—	1	—
Rheumatoid arthritis	1	1	—	1	—	—
Total	27	27	8	11	2	5
			1 Improving			

### CONCLUSION

Data from such limited number of cases cannot guarantee an absolute efficiency of auto-uro-proteose therapy. On the other hand, allergy being a complex phenomenon, a single remedy seems impracticable. The clue from some of the dramatic effects of skin-testing on the symptom-complex should not be ignored and every effort should be made to explain apparent improvements scientifically in some of the cases. Relapses in this series should not stand in the way of further investigation on the allergenic character of the urinary proteose. In fact, relapses should be expected, if the hypothesis of hypo-sensitisation therapy depends on the feasibility of effective elimination of the allergen from the patient's environment during course of treatment. Such a technique in secondary endogenous allergies may therefore, be destined with disappointing results as elimination of allergen during the therapy is not always possible. Moreover, clinical improvements with hypo-sensitisation does not necessarily mean actual sensitiveness of the organism to the noxious agent, nor does it guarantee massive or frequent exposures to allergen with

impunity. With this possible explanation of the relapse in the case of auto-uro-proteose therapy investigation on the urinary proteose on scientific lines is worth trying.

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NATURE AND SPECIFICITY OF URINARY PROTEOSE  
PART II. DEMONSTRATION OF ANTIBODIES AGAINST URINARY  
PROTEOSE, USING IT AS AN ANTIGEN

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Allergic state, according to modern conception, is basically an antigen-antibody reaction. Recognition of an allergen, the diagnosis of an allergic state and even the treatment of the condition are often planned in terms of antibodies, produced by allergen, in the system of the host. Demonstrations of these antibodies have been tried in various allergic states but so far our laboratory techniques have proved not sufficient to detect this antigen-antibody reaction in every case. Difficulties have not always been with the technique alone but sometimes due to non-availability of the antigen causing sensitised reactions. Precipitin-test against some of the exogenous agents, particularly in food allergies, has been possible, but these antibodies are also present in individuals consuming that particular article without showing any hypersensitiveness to it. This reaction cannot, therefore, distinguish the pathological sensitisation from a physiological defensive response. Urbach (1), by modern technique of Cannon and Marshall, demonstrated specific precipitins in sera from patients known to be hypersensitive to egg-protein, tuberculin and crystalline insulin. Position of the complement-fixing antibodies to egg-white in hypersensitive children demonstrated complement-fixing antibodies to egg-white only showed complement deviation with concentrated antigen. Jaffe has demonstrated complement-fixing antibodies to fish, yeast and lentils, so also Urbach to egg, milk and pollens. They, however, advocate that with the development of the test, detection of these allergic antibodies might be a possibility in every clinical condition. Besides this inefficiency of the immunological methods in detecting such a delicate antigen-antibody reaction, availability and approachability of antigen afford a special difficulty, particularly in endogenous and drug allergies, as in such conditions intermediary or conjugate products act as antigens and we neither know their exact nature, nor can we isolate them by existing methods. Any technique which will isolate or identify these secondary allergens will considerably revolutionize the study *in-vitro* of the antigen-antibody reaction in these clinical entities.

URINARY PROTEOSE AS AN ALLERGEN

Oriel's (2) hypothesis on allergenic character of urinary proteose is mainly based on Manwaring's (3) observations that "marked chemical alterations take place in specific antigens after introduction and that many of the immunological adaptations are to the resulting denaturation products (secondary antigens) rather than the primary antigens originally injected". Proteose in the urine, according to Oriel, is

such a denaturation product of some primary antigen, but how and by what organ this is caused is not definitely known. He accuses the liver as a prime organ in the production of proteose, a secondary allergen. Importance of liver in the production of experimental anaphylaxis in dogs has been duly demonstrated by Dale (4) and Manwaring *et al* (5) ; but production of histamine alone under experimental conditions cannot explain all the findings observed, e.g., diminished coagulability of blood. Furthermore, "it is unlikely that histamine which the liver must pour in the general circulation under such condition will be free: it is more likely to be involved in colloidal complexes with other cell constituents" (Dale). Oriel regards urinary proteose as this complex substance produced by allergised liver cells in response to the exposure of primary agents. It contains the factor to which the patient is sensitive and probably it is the denaturated product of the primary antigen either explosively formed or explosively liberated from the store house liver. Urbach (1) also supports Oriel's views. He regards urinary proteose as a secondary endogenous allergen. But mere acceptance cannot establish this as such scientifically. An allergen must satisfy three objective conditions: (i) that it can be effectively used in hypo-sensitisation therapy with clinical improvements, (ii) that antibodies can be demonstrated against such suspected substance, and (iii) that passive transference test with the substance is possible. Clinical trial with proteose therapy suggests that urinary proteose is in the same way related to the factor to which the patient is sensitive. The present note will deal with the possibility of demonstration of auto-antibodies against urinary proteose from various allergic cases.

#### CASES OF STUDY

Urinary proteose from various endogenous allergic cases were tested. Most of the cases were asthmatic while among others were cases of psoriasis, urticaria, neuro-dermatitis and arthritis. Taking tuberculosis to be essentially an endogenous allergic disease, several cases of pulmonary tuberculosis were also studied along with this series of allergic conditions. As control normal sera were tested. The nature of the study being most preliminary, cases were stray and unselected.

#### EXPERIMENTAL

The technique of antigen-antibody reaction in this study has been based on the method of complement-fixation reaction, as described in the British Medical Research Committee Report (1918) on Wassermann Reaction with slight modifications. Main principles of the procedure will be discussed in the note.

#### REAGENTS

CHART I

Animal	Antigen.	Serum dilution 1 in						
No.		10	20	50	100	250	500	1000
1	7	++++						
2	11 (T)	+++	+++	++	+	—	—	—
3	22	+++	+++	+	+	?	—	—

— indicates no inhibition of hæmolysis.  
+ indicates inhibition of hæmolysis.

For anticomplement tests: 10 and 20 for antigen, 100 for antiserum.

— indicates no inhibition of hæmolysis.  
+ indicates inhibition of hæmolyses.

The titration of the antigen with human serum could not be relied on, as the entire hypothesis of the experiment might be in the melting pot, while immune animal sera with fairly high antibody titre could not be utilized in settling the titre of the antigen to be used in the tests with human sera. The working dilution of the antigen was, therefore, arbitrarily selected by its anti-complementary value. Double dilution of the strength showing no anti-complementary effect on sensitized sheep's R.B.C. with 1 m.h.d. complement was accepted as the antigenic titre in the test. In this series the working dilutions in most cases were  $1/2$  of original strengths of proteose emulsion.

Sera to be tested were collected aseptically from starving patients, inactivated at 56°C for half-an-hour and then stored in ice box overnight. Since freshly inactivated samples may show an increased liability for i's reactive substances and therefore might yield false serological findings, the tests were not carried out on the day of inactivation. Dilution of serum in tests proper was 1:5. Pooled normal guineapig-sera were used as the source of complement, original titre varying from 1/40 to 1/60. The working dilution of the complement was 2 m.h.d. The haemolytic system was 3% sheep R.B.C. suspension (51% haemoglobin) sensitized with 0.5 m.h.d. amboceptor (titre 1/2,000).

## MAIN TEST

MAIN TEST

In the main test 6 tubes were used for each serum tested. The first tube which was used for serum control contained no antigen, the second and the third contained all the reagents with different amount of serum. Fourth served as an antigen control with no serum, the fifth to examine the haemolytic property of the antigen and the last or the sixth to test the haemolytic system. In a typical positive result,

accepted in this series first, fourth and the sixth showed complete hæmolysis while the second and the third presented inhibition of hæmolysis to the degrees depending on the strength of reactions. The fifth being hæmolytic control, showed no trace of hæmolysis. The amounts of reagents added to the tubes in carrying out the tests are given below:

Tube No. 1—Serum (1/5) 3 volumes, complement 1 volume (2 m.h.d.), normal saline 1 volume.

Tube No. 2—Serum 2 volumes, complement 1 volume, antigen (1/2) 1 volume and saline 1 volume.

Tube No. 3—Serum 3 volumes, complement 1 volume and antigen 1 volume.

Tube No. 4—Antigen 2 volumes, complement 1 volume and saline 2 volumes.

Tube No. 5—Antigen 3 volumes and saline 2 volumes.

Tube No. 6—Saline 4 volumes and complement 1 volume.

The whole set was then kept at 37°C for 45 minutes in an incubator, then in a cold chamber at 22°C for 15 minutes. 1 volume of hæmolytic system was added to each tube and finally incubated at 37°C till the control tubes showed complete hæmolysis. After noting the preliminary reading of hæmolysis in each tube, the set was kept overnight in a frigidaire to allow R.B.C. to settle at the bottom. Final reading was taken on the next day.

#### INTERPRETATION OF RESULTS.

Results were denoted by + and - signs. + + + + indicates strong inhibition with precipitate of R.B.C. at the bottom of the tube and crystal clear supernatant fluid, + + + as fairly strong inhibition with a tinge of colour in the supernatant fluid, + as a definite inhibition with a precipitate of R.B.C. but fair colouration of fluid column. Trace reaction (tr.) was indicated by a distinct precipitate of R.B.C., but moderate colouration of the fluid. Doubtful reactions were recorded by ± signs while negative ones by - signs. Upto + + signs results were taken to be positive while + and trace reactions as weakly positive findings. Results of complement-fixation tests so far done are tabulated in the following protocols.

#### CHART II

*Complement-fixation tests on allergic cases with urinary proteose as antigens.*

Dis. ase.	No.	Reactions in tubes
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have also been studied to detect any false-positive results. Number of cases under scrutiny is very limited and results are as follows:—

CHART IV

*Cross-reactions with tubercular and allergic proteose as antigens.*

Serum.	No.	Antigens.		Reactions in tubes.					
				1	2	3	4	5	6
Tubercular	5	Allergic proteose	5	—	—	—	—	++++	—
Allergic	5	Tubercular proteose	1	—	±	±	—	++++	—
			4	—	—	—	—	++++	—
Normal	3	Both	3	—	—	—	—	++++	—

To facilitate comparison the above tables may be summarised as follows:—

CHART V

*Complement-fixation test with urinary proteose as antigen.*

Antigen used in the tests.	Nature of cases.	No. of cases.	Results		
			Positive	Doubtful	Negative
Auto-proteose	Allergic cases	11	7	Nil	4
Tubercular proteose	Tubercular allergy	40	30	Nil	10
	Non-tubercular allergy	4	Nil	Nil	4
	Normal cases	3	Nil	Nil	3
Hetero-proteose	Tubercular allergy	5	Nil	Nil	5
	Non-tubercular allergy	5	Nil	1	4
	Normal cases	3	Nil	Nil	3

### CONCLUSION AND SUMMARY

Therapeutic trials with urinary proteose suggest some specific property to the substance. Charts I to IV will reveal that beneficial effects in clinical conditions are probably due to its antigenic property. Antibodies seem to be specific to the proteose and it will be shown in later publications that specificity is due to incorporation into its structure of bacterial hapten to which the patient is sensitive.

NATURE AND SPECIFICITY OF URINARY PROTEOSE.  
PART III. SPECIFICITY OF URINARY PROTEOSE AND THE BACTERIA  
SUSPECTED TO BE THE PRIMARY FACTOR FOR THE CONDITION.

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Previous notes on the subject seem to have revealed that urinary proteose has some therapeutic effect as hyposensitising agent in suitable cases. Its effect may be due to its antigenic nature, as antibodies have been demonstrated in patients and have been actively produced in laboratory animals. All these findings may show its allergic nature but cannot express its relation, direct or indirect, with the primary agents causing the elaboration of such a secondary product. Specificity of anti-proteose antibodies has been observed, but what determines its specificity is a problem for investigation. If bacterial or any other microbial metabolism has anything to do with the production of urinary proteose, a secondary endogenous allergen, it

may be expected that these microbial agents or their metabolites directly influence the specificity of urinary protease. This piece of work has been planned to scrutinise and detect any such role of the microbial agent in shaping the form and the character of the urinary protease.

#### PLAN OF THE INVESTIGATION

Identification of the bacterial entity of the urinary protease in allergic cases has been planned on the basis of Landsteiner's (1) views that proteins lend to their respective antibodies absolute specificity. These specificities of proteins depend on the "determinant" or "hapten" linkages specific to them, so that they may be effectively identified in terms of their antibodies. If, in the case of urinary protease, bacteria have at all any function to determine its specificity, it is probably by such haptensisation of the urinary protease by bacterial haptens or antigens. Anti-protease-antibodies may, therefore, be expected to exhibit such a relation with bacterial antigens, which is regarded to orient its specificity.

In this piece of investigation anti-protease sera were produced in laboratory animals, rabbits and guineapigs. These anti-sera were cross-matched with different bacterial antigens by complement-fixation reactions. Modification of the technique has often been tried with a view to improve the efficiency of the tests. In the first part of the investigation bacterial emulsions were used as antigens, but as bacterial proteins are better antigens than emulsions or lysates, in the later portion bacterial proteins were used instead of emulsions. The reactions observed in the series being weak, the technique was further modified and tests were carried out at a lower temperature.

#### EXPERIMENTAL DETAILS

*Production of anti-protease serum in the rabbits :*

To 100 cc. of sterile normal saline, containing 200 millions organisms per cc., was added 1 cc. of N/1 NaOH. The whole mass of liquid was then put on a waterbath at 37°C for 2 hours. To this lysed bacterial emulsion was then added 10% trichloroacetic acid till fine flocculations occurred (usually 2 to 2.5 cc.). The flask was then put on the bath at 37°C for  $\frac{1}{2}$  hour. The protein-floccules were then centrifuged and the precipitate dissolved in saline, 4 cc. per 100 cc. of original emulsion. N/10 NaOH was then added drop by drop till the precipitate went to colloidal solution. 1 cc. of 1 per cent formol-saline was then added as preservative. The final pH of the antigen-solution was adjusted between 7.6 to 7.8.

Anti-complementary properties and antigenic qualities of these bacterial protein-antigens were tested, as in previous paper (3) before employing them as antigens in tests proper with anti-proteose-serum. Anti-complementary dilutions of these protein-antigens were streptococcus 1:4 in 1.5 m.h.d., micrococcus catarrhalis 1:4 in 1.5 m.h.d., pneumococcus 1:4 in 1 m.h.d. and Friedlaenders pneumo bacillus 1:4 in 1 m.h.d., the complement titre being 1:40. 1:20 dilutions of these antigens were capable of combining with respective antibodies. None of these showed any hæmolytic tendencies. Working dilutions of these antigens were, streptococcus 1:10, micrococcus 1:10, pneumococcus 1:5 and pneumo bacillus 1:5. Old tuberculin was used to serve the purpose of tuberculo-antigens in dilution of 1:50, as it contains the tubercular hapten.

#### *Main test :*

The technical details of the test proper were same as given in the previous paper (3) on the demonstration of antibodies against urinary proteose. The working dilution of serum was 1:5 and complement was used in 2 m.h.d. The control tubes for serum and antigens contained 1 m.h.d. complement and the hæmolytic control tubes for antigen contained 3 volumes of antigen with no complement. The set was incubated at 37°C for 1 hour and then at 22°C for 15 minutes. Hæmolytic system was then added in each tube and readings were taken after the control tubes showed complete hæmolysis. The results of the tests may be evidenced from the protocols I and II.

#### *Complement-fixation reaction at lower temperature :*

The results observed, though revealing a number of positive findings, were not very clear cut, probably due to weak antigen-antibody reaction *in-vitro*. It was, therefore, tried to modify the technique of the test in such a way as to suit most efficiently the delicacy of the reactions. Complement-fixation reaction at lower temperature, as outlined by Russian workers (4) for testing typhus-sera, was given a trial with some modification of original technique.

*Working dilution of the complement :*

Guineapig's serum was the source of complement. Different dilutions of complement, 1 in 10, 1 in 15, 1 in 20, 1 in 25, 1 in 30, 1 in 40, 1 in 50, and 1 in 60, were made with normal saline. 0.2 cc. of each dilution was titrated with 0.4 cc. each of normal saline and hæmolytic system containing 3 per cent suspension of sheep's R.B.C. and 5 m.h.d. anti-sheep amboceptor. Readings were taken after incubation at 37°C for  $\frac{1}{2}$  hour. Working dilution of complement was fixed at the dilution in the second tube from that indicating the titre of complement, that is, if the titre was 1:25, the working dilution of complement was 1:15.

*Determination of complement unit :*

Complement in working dilution was then titrated with the reagents, serum and antigens ; 7 tubes for each reagent were taken. Complement in that dilution was then added in quantities, 0.06 cc., 0.07 cc., 0.08 cc., 0.09 cc., 0.1 cc., 0.15 cc., and 0.2 cc. 0.2 cc. of normal saline was added in each tube. After addition of 0.2 cc. of reagent, serum or antigen, the tubes were incubated at 37°C for one hour ; 0.2 cc. of hæmolytic system was then added to each tube and reading was taken after 45 minutes' incubation at 37°C. Each reagent was titrated separately for its respective complement-unit. *Complement-unit (c.u.) for a particular reagent was taken to be the lowest quantity of complement-dilution producing complete hæmolysis of 0.2 cc. of hæmolytic system after 45 minutes' incubation, and the complement-unit for the main test (with both serum and antigens) was the lowest common quantity of complement giving complete hæmolysis of the given quantity of hæmolytic system when titrated with reagents separately ;* taking for instance, if the complement-unit for streptococcal antigen was 0.09 cc. and that for the anti-proteose serum 0.06 cc., the complement-unit for the main test with streptococcal antigen and anti-proteose serum was 0.09 cc. Higher multiples of the unit, such as, 1.5 complement-unit, 2.0 complement-unit, 2.5 complement-unit etc., could be accordingly calculated

*Main test :*

In the main test, 0.2 cc. serum and 0.2 cc. antigen were taken in small sugar tubes, to them were added different complement-units, usually 1 c.u., 1.5 c.u., 2 c.u. and 2.5 c.u. 0.2 cc. normal saline was used to replace serum in antigen-control tubes and antigens in serum-control tubes. At the utmost, therefore, 16 tubes were scheduled for each antigen, 4 for serum control, 4 for antigen control, 4 for anti-serum with the antigen and 4 for normal serum with the antigen. The whole set of tubes was then placed inside a refrigerator (temperature 0 to 4°C) for 18 hrs. After the cold incubation, 0.2 cc. of hæmolytic system was added to each tube and the tubes incubated at 37°C. The readings were taken as soon as the controls showed complete hæmolysis. Bacterial proteins were used as antigens and the working dilutions were as in the tests at 37°C (vide supra). The results are given in the pages of III.

*Interpretation of results :*

Results in the tables have been indicated as in previous paper by + and - signs, + indicating positive and - negative reactions. Different gradations of positive reactions have been represented by varying numbers of + signs, complete inhibition of hæmolysis by + + + +, definite inhibition by + signs, while inhibition in trace by tr.

Findings of the tests seem to indicate that proteose has a relation with bacterial antigens and the observed specificity of urinary proteose may be due to a probable haptisation of proteose by bacterial antigen. These serological results have also been corroborated by other observations in some of the cases. A case of neuro-dermatitis (P7)\* showing bacterial entity of urinary proteose to be streptococcal, was given intravenous streptococcal vaccine with rapid clinical improvement. Subsequently, penicillin in high doses was prescribed and the skin condition has further improved. A case of pleurisy (P 24) seemed to have excreted tubercular proteose. Pleural fluid from the case was injected into a guineapig which died with typical tubercular lesions on liver, lung and abdominal lymph glands. Proteose from a case of psoriasis (S.T.M.) seemed to contain tubercular antigen. The same case developed acute pleurisy during the course of investigation. Anti-serum against proteose sample from a case of suspected tubercular adenitis (P38) did not give any cross-fixation with tubercular antigen. Aspirated fluid from the glands was injected into a guineapig which is still healthy (three months after injection). Proteose samples from a case of rheumatoid arthritis (P28) and a case of allergic conjunctivitis (P42) seemed to contain micrococcal antigen. The cases were tested for micrococcal sensitiveness by skin test. They developed marked immediate wheal to intracutaneous injection of 0.1 cc. of 1 million organisms per cc., but not to streptococcus or staphylococcus obtained from their throats. These findings, therefore, tend to express that the proteose has some bacterial entity. As observations on a small number of cases cannot establish such an important fact, work is being continued to develop this technique of identifying bacterial entity of urinary proteose; if scientifically established, such a procedure may in future be employed in the investigation of various clinical conditions of unknown etiology, in estimating and assessing the amount of allergic state in an individual particularly in tubercular conditions and in understanding two distinct, yet unintelligible phenomena—the anti-bacterial immunity and anti-bacterial allergy in human system.

## CONCLUSION AND SUMMARY

Anti-proteose serum in rabbits gave cross-fixation with bacteria a responsibility of bacteria in the elaboration of urinary proteose is corroborated by other clinical investigations. Specificity of urinary proteose to the authors, is probably due to haptisation of proteose by bacteria.

\* 'P' stands for "proteose". P7 means "proteose No. 7".

## PROTOCOL I

*Complement-fixation reaction with bacterial emulsion and protease as antigens.*

Complement-fixation reactions with antigens												Protease
Case No.	Date	Serum No.	Strep. Mic. cat. Pneumo. Friedl. B. coli. Salmonella T50.							(stock strain)		
Neurodermatitis P <sub>7</sub>												P7
bronchary tuber- culosis; P <sub>11</sub>												P11
cystitis, P <sub>23</sub>												P23
cystitis, P <sub>24</sub>												P24
testinal tuber- culosis; P <sub>25</sub>												P25
bronchary tuber- culosis; P <sub>26</sub>												P26

Case. No.	Date.	Serum No.	Complement-fixation reactions with antigens						Proteose		
			<i>Strep.</i>	<i>Mic. cal.</i>	<i>Pneumo.</i>	<i>Friedl.</i>	<i>B. coli.</i>	<i>Salmonella</i>		<i>T50.</i>	
			(stock strain)								
Parotitis (S.T.M.): P <sub>27</sub>	5-10-48	16 R	+	—	—	—	—	+	P <sub>24</sub>	P <sub>26</sub>	P <sub>27</sub>
		N (normal)	—	—	—	—	—	—	—	—	+
	19-10-48	16 R	+	—	—	—	—	+	+	+	+
		N (normal)	—	—	—	—	—	—	—	—	—
Rheumatoid arthritis, P <sub>28</sub>		17 R	—	+	—	*	*	*	P <sub>28</sub>		
		N (normal)	—	—	—	*	*	*	+	+	+
Rheumatism, P <sub>31</sub> Arthritis, P <sub>38</sub>	6-5-49	20 R	tr.	—	—	*	*	—	P <sub>34</sub>	P <sub>38</sub>	
		21 R	—	tr.	—	*	*	—	+	+	+
		N <sub>1</sub> (normal)	—	—	—	*	*	—	+	—	—
		N <sub>2</sub> (normal)	—	—	—	*	*	—	—	—	—
+ indicates inhibition and — indicates no inhibition.											

+ indicates inhibition and — indicates no inhibition.



PROTOCOL II

*Complement-fixation reaction with bacterial proteins and protease as antigens.*

Date	Anti-serum.	Results of complement-fixation reaction with antigens				
		Bacterial protein		Protease		
		<i>Strepto.</i>	<i>Mic. cat.</i>	<i>Pneumo.</i>	<i>Friedl.</i>	
13-5-49	20 R	—	—	—	—	P38 +
	21 R	—	+	—	—	+
	N <sub>1</sub> (normal)	—	—	—	—	+
	N <sub>2</sub> (normal)	—	—	—	—	—
	N <sub>3</sub> (normal)	—	—	—	—	—
20-5-49	20 R	+	—	—	—	P34 + + +
	21 R	—	+	—	—	+
	N <sub>1</sub> (normal)	—	—	—	—	tr.
	N <sub>2</sub> (normal)	—	—	—	—	—
	N <sub>3</sub> (normal)	—	—	—	—	—
1-6-49	20 R	+	—	—	—	+
	21 R	—	tr.	—	—	+
10-5-49	26 R	—	tr.	—	—	P39 + +
	N (normal)	—	—	—	—	P40 + —
21-6-49	27 R	—	±	—	—	+
	N (normal)	—	—	—	—	+
21-6-49	27 R	—	—	—	—	+
	N (normal)	—	—	—	—	+
1-7-49	27 R	—	—	—	—	+
	28 R	—	+	—	—	+
21-6-49	28 R	—	—	—	—	+
	N (normal)	—	—	—	—	+

— indicates inhibition and — indicates no inhibition.

PROTOCOL III.  
*Complement-fixation reactions at lower temperature with bacterial  
protocose as antigens.*

c.u.	+	-	—	+	—
ur.	—	—	—	+	—

c.u. = complement-unit; + indicates inhibition and — indicates no inhibition.

derivatives of sulphacetamide, sulphabenzamide, sulphadiazine, sulphamerazine and sulphamethazine were prepared in this laboratory by reacting either with formalin or hexamine to afford the corresponding p-hydroxy methylaminobenzene sulphonamides (Basu, 19). All the compounds have been designated in short as "Formo" derivatives. The present paper deals with the *in-vitro* activity of these compounds against *Vibrio cholerae*. Comparison of their activity has been done with a product similar to the compound "6257" (Bhatnagar, *loc. cit.*).

### EXPERIMENTAL

All the compounds were tested in the form of emulsion in 0.2% tragacanth solution. A stock solution of 100 mg. per cc. was first made, and requisite quantities were added from this stock emulsion to the test media, to give the required concentration for the drug under study. Tests were put up in 10 cc. each of 1 per cent. peptone water (Difco), glucose broth, and papain digest broth. The drug-charged media tubes were sterilized under 10 lbs., pressure for 20 minutes and then inoculated with one loop (1 mm.) of a suitably diluted culture of smooth strains of *V. cholerae*, (*Inaba* and *Ogawa*). The inoculum consisted of about 500 to 1000 cells checked by plate counting. Tables I, II and III summarise the results of these experiments. By bacteriostasis is meant the concentration of the drug which prevented visible turbidity of growth up to 24 or more hours. Bacteriolytic concentration has been determined by the absence of growth when the tubes showing bacteriostasis were sub-cultured after 24 hours.

TABLE I

*In-vitro anti-vibrio-cholerae activity of "Formo"-sulphonamides in 1 per cent peptone water.*

*Inoculum 500-1000 cells, Incubation 37-38°C.*

Figures indicate milligrammes per cc.

Compounds.	Minimum bactericidal concentration against.		Minimum bacteriostatic concentration against.	
	<i>Inaba</i>	<i>Ogawa</i>	<i>Inaba</i>	<i>Ogawa</i>
1. Formo-sulphathiazole '6257'	4.0	4.0	1.0	1.0
2. Formo-sulphacetamide (S A F.)	2.0	2.0	1.0	1.0
3. Formo-sulphabenzamide (S B F.)	2.0	2.0	1.5	1.5
4. Formo-sulphadiazine	3.0	3.0	1.5	1.5
5. Formo-sulphamerazine	3.0	3.0	1.5	1.5
6. Formo-sulphamethazine	3.5	3.5	1.5	1.5

TABLE II

Showing the bacteriostatic activity of compounds in 10 cc. glucose broth.  
Incubation 37-38°C. Inoculum 500-1000 cells (by count) of *V. cholerae*.

Drug.	Concentration in media. mg. per cc.	Observations							
		<i>V. cholerae</i> Ogawa.				<i>V. cholerae</i> Inaba.			
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
Formo-sulphacetamide	5.0	—	—	—	—	—	—	—	—
	2.0	—	—	—	—	—	—	—	—
	1.0	—	—	—	—	—	—	—	—
	0.5	+	+	+	+	—	+	+	+
Formo-sulphabenzamide	5.0	—	—	—	—	—	—	—	—
	2.0	—	—	—	—	—	—	—	—
	1.0	—	—	—	—	—	—	—	—
	0.5	+	+	+	+	—	+	+	+

+ = growth ; — = no growth.

TABLE III

Showing the bacteriostatic activity of drugs in 10 cc. of papain digest broth.  
Inoculum about 500 cells of *V. cholerae*. Incubation 37-38°C.

Drug.	Concentration in media. mg. per cc.	Observations							
		<i>V. cholerae</i> Inaba.				<i>V. cholerae</i> Ogawa.			
		24 hrs.	48 hrs.	72 hrs.	96 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
Formo-sulphacetamide	2.0	—	—	—	—	—	—	—	—
	1.0	—	—	—	—	—	—	—	—
	0.5	—	—	—	—	—	—	—	—
Formo-sulphabenzamide	2.0	—	—	—	—	—	—	—	—
	1.0	—	—	—	—	—	—	—	—
	0.5	—	—	—	—	—	—	—	—

+ = growth ; — = no growth.

It may be seen from Table III that the bacteriostatic potency of both the drugs, Formo-sulphacetamide and Formo-sulphabenzamide, against Inaba and Ogawa sub-types have varied widely when the tests are run in papain-digest broth. The cause may lie in the production of greater quantity of para-aminobenzoic acid by the Ogawa vibrio in this medium. It is also possible that the "Ogawa" strain is actually more resistant to the drugs than the "Inaba." A few experiments done according to the solid streak-agar method (Table IV) demonstrate such difference in susceptibility of the two strains.

TABLE IV

*Showing the bacteriostatic activity of drugs in nutrient agar by the solid streak-agar method.*

Incubation at 37°-38°C. Reading after 24 hours.

Drug.	Concentration in agar mg.	Observation	
		<i>Inaba.</i>	<i>Ogawa.</i>
Formo-sulphacetamide	335	—	—
	266	—	+
	100	+	+
Formo-sulphabenzamide	336	—	—
	266	—	+
	100	+	+

+ = growth ; — = no growth.

#### RESULT OF OBSERVATION

From Table I it may be seen that both Formo-sulphacetamide and Formo-sulphabenzamide exert a greater bactericidal potency than the rest of the compounds tested so far. Their bacteriostatic potencies are also high, but those of Formo-sulphacetamide and Formo-sulphathiazole appear to be of the same order. The formalin derivatives of the other sulphonamides e.g. sulphadiazine, sulphamerazine, and sulphamethazine, appear to have a lower range of both bacteriostatic and bacteriolytic effect. Thus, while Formo-sulphacetamide and Formo-sulphathiazole give bacteriostatic effect at 100 mg. % concentration, Formo-sulphabenzamide exerts the same at 150 mg. % and the diazine, merazine and methazine derivatives exert at only a concentration of 250 mg. % in media. In papain-digest broth Formo-sulphacetamide exerts slightly more powerful activity against 'Inaba' than Formo-sulphabenzamide (Vide Table III). In agar-strip as well as in glucose broth, each of these compounds exerts identical activity.

An interesting observation has been made on the activity of the "formo" derivatives in this connection. If the drugs are tested by dissolving them in alkali, they lose some of their bacteriostatic activities. They are more active when added directly into the media.

#### DISCUSSION

be more efficacious. Cholera is a disease with less systemic involvements. But while the whole of the intestinal tract is involved, other affections such as of the gall-bladder and the kidneys are sometimes common associated factors. It is known that Cholera vibrios can enter the portal circulation and are found to be excreted in the gall bladder. It is, therefore, expected that for counteracting the systemic effects of the disease, absorption of potent drugs might play a part. It appears (25) that whatever activity is shown by Sulphaguanidine in intestinal diseases is dependant on its high dosage, which ensures proper systemic absorption apart from high local concentration.

The presence of the sulphonamides in active anionic form plays an important part in effective bacteriostasis (26). It is expected that the free amino grouping would then be in a position to block the carboxyl groups of the bacterial enzyme proteins, thus leading to a reversible combination. That sulphonamides combine with proteins have been amply demonstrated by Davis and Wood (27). Therefore, the hydroxy-methyl derivatives ("Formo"-derivatives) being compounds readily dissociating into the free anionic form may be expected to exert powerful therapeutic action, so far as bacteriostatic and bacteriolytic properties are concerned (Table I). S.A.F. and S.B.F. having greater bactericidal effect are liable to show greater therapeutic effectiveness *in vivo*. Toxicological studies, so far made (unpublished data) show that both these are well-tolerated drugs, though in acute toxicity experiments, S.A.F. shows a still lower toxicity. Moreover, the slow liberation of formaldehyde in the system may enhance its local anti-bacterial action in the intestines and the gall bladder. The compounds, during excretion through the kidneys, are liable to be further broken up at acid pH of the urine, liberating fairly good quantities of formaldehyde and the free sulphonamide derivative which would be helpful in tackling the secondary genito-urinary invaders. Thus conditions of associated pyelitis, cystitis with cholera may be usefully counteracted.

It has been shown (Bose and Ghosh, 11) that the efficacy of sulphanilyl-benzamide in bacillary dysentery depends to a great extent on its selective re-excretion through the caecum and large intestines, the parts affected in bacillary dysentery. It is expected that Formo-sulphabenzamide when broken down into free Sulphanilyl-benzamide in the system, would be similarly liable to be excreted into the large intestines, thus enhancing the local effect of the drug. Whether the other compounds under identical conditions will have the same type of selective re-excretion, has yet to be seen. It can however, be concluded from the studies so far made that the formaldehyde derivatives of Sulphacetamide and Sulphanilyl-benzamide merit further trials in the clinical field.

#### SUMMARY

1. The formaldehyde condensation products of Sulphathizole (S.T.F.), Sulphacetamide (S.A.F.), Sulphanilyl-benzamide (S.B.F.), Sulphadiazine, Sulphamethazine and Sulphamethazine have been tested *in-vitro* against *Vibrio cholerae*.
2. The "Formo"-derivatives of Sulphacetamide and Sulphanilyl-benzamide appear to exert better bactericidal effect than that exerted by the others.

3. Clinical evaluation of the condensation products, particularly S.A.F. and S.B.F. is suggested on the basis of experimental results.

My thanks are due to (Late) Mr. N. Rao for part of the data in Table I.

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## STUDIES ON THE CHEMOTHERAPY OF BACTERIAL INFECTIONS: PART II. *IN-VITRO* SCREENING OF CERTAIN FORMALDEHYDE SULPHONAMIDE DERIVATIVES AGAINST VARIOUS PATHOGENS

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In Part I of this series, anti-bacterial activity of the respective formaldehyde-condensation products of Sulpha-thiozole (S.T.F.), Sulphacetamide (S.A.F.), Sulphabenzamide (S.B.F.), Sulphadiazine, Sulphamerazine, and Sulphamethazine against *Vibrio cholerae* have been studied. The compounds S.A.F., S.B.F. and S.T.F. being more promising, they have been taken for further screening tests against various pathogenic organisms. The present paper deals with the bacteriostatic activities of these compounds against *staphylococcus*, *pneumococcus*, *E. typhosa*, *S. paratyphi A*, *Sh. dysenteriae* (Shiga), *Sh. sonnei*, *E. coli*, *B. proteus vulgaris*, and *B. pyocyaneus*.



## EXPERIMENTAL

All the bacteriostatic tests were performed either in 1% Difco peptone water media or in hormone broth according to the procedure already reported (1). For comparison, sulphathiazole was also studied. The inoculum used in all the tests was high, being 1 loopful (1 mm.) of an 18 hours' liquid culture of the different organisms. Each screening experiment was repeated thrice. In every experiment three individual tubes containing the same test dilution of the drugs were charged, so that in total nine experiments were performed for each concentration of the drug. Results were noted only when six out of the nine tubes gave the same results. The observations were recorded after 48 hours.

Tables I to III summarise the results of these experiments.

TABLE I

*Showing the in-vitro bacteriostatic activity of "formo"-sulphonamide derivatives in comparison with sulphathiazole. 10 cc. hormone broth media, pH 7.4. Inoculum 1 loopful (1 mm.) of organisms from 18 hours' growth in hormone broth culture medium. Incubation 37-33°C.*

Drug.	Concentration in media. mg. per cc.	Micro-organism		
		<i>Streptococcus</i> <i>hemolyticus</i> (Richards)	<i>Streptococcus</i> <i>viridans</i> 36r	<i>Pneumococcus</i> Type I
Sulphathiazole	1.0	+	+	+
	2.0	+	+	—
	2.5	+	+	—
S.A.F.	1.0	+	+	+
	2.0	+	+	—
	2.5	—	—	—
S.B.F.	1.0	+	+	+
	2.0	+	—	—
	2.5	—	—	—
S.T.F.	1.0	—	+	+
	2.0	+	+	—
	2.5	—	+	—

TABLE II

Showing the bacteriostatic activity of "formo"-sulphonamides in comparison with sulphathiazole against various intestinal organisms in 1% peptone water media, pH 7.6. Inoculum—1 loopful (1 mm.) of 18 hours' culture in peptone water.

Drug.	Concentration in media mg. %	Microorganisms			
		<i>S. typhi</i> O	<i>Para-typhi</i> A	<i>Sh. Dysen-</i> <i>teriae</i> ( <i>Shiga</i> )	<i>Sh. Sonnei</i>
Sulphathiazole	5.0		x	+	x
	12.5		x	—	x
	25	x	x	—	x
	50	±	+	—	+
	100	—	+	—	—
	200	—	—	—	—
S.A.F.	5.0	x	x	+	
	12.5		x	—	
	25		x	—	
	50	+	+	—	+
	100	—	—	—	—
	200	—	—	—	—
S.B.F.	5.0		x	+	x
	12.5		x	—	x
	25			—	
	50	+	+	—	+
	100	—	—	—	—
	200	—	—	—	—
S.T.F.	5.0	x	x	+	x
	12.5	+	x	—	x
	25	+	x	—	.
	50	±	+	—	x
	100	—	+	—	-
	200	—	—	—	—

± = Partial inhibition of growth.

+

— = Inhibition of growth.

x = Not done.

TABLE III

Showing the bacteriostatic activity of "formo"-sulphonamides in comparison with Sulphathiazole against the pathogens of the genito-urinary tract in 2% Peptone water media, pH 7.6, Inoculum—1 loopful (1 mm.) of 18 hours peptone water culture.

Incubation 37-38°C							
Drug.	Concentration in media mg. per cc.	Microorganisms					
		Staph. <i>aureus</i>	<i>E. coli</i>	<i>B. proteus</i> 387    ox2		<i>B. pyocyaneus</i> 128    308	
Sulphathiazole	3.0						+
	2.0	+	—	—		+	+
	1.5		+	+	—	+	
	1.0	+	+	+	—	+	
	0.5	+	+	+		+	
Formo-Sulphacetamide	2.0	—	—	—		—	
(S.A.F.)	1.5	x	x	—		—	—
	1.0	+	—	—	—	+	+
	0.5	+	+	+	—	+	
Formo-Sulphabenzamide	2.0	—	—	—		—	
(S.B.F.)	1.5			—		—	
	1.0	—	—	—	—	—	—
	0.5	+	+	+	—	+	+
Formo-Sulphathiazole	2.0	+	+	+	+	—	
(S.T.F.)	1.5		x	+		+	
	1.0	+	+	+		+	
	0.5	+	+	+		+	

+ = No inhibition of growth.  
— = Inhibition of growth.  
x = Not done.

DISCUSSION

Thus, from Table I, we may infer that the formo-derivatives of sulphacetamide (S.A.F.) and sulphabenzamide (S.B.F.) are likely to be more effective against *streptococcus viridans* infection than Sulphathiazole and "Formo"-sulphathiazole (S.T.F.). Even against *staphylococcus aureus*, S.A.F. and S.B.F. appear to be more bacteriostatic (vide Table III), S.B.F. being still more powerful than S.A.F. in this respect. Against the urinary pathogens, *E. coli*, and *B. proteus*, S.T.F. seems to fail in showing definite bacteriostatic action while S.A.F. and S.B.F. exert fairly high and similar effect, both appearing to be more potent in these infections than sulphathiazole. Against *B. pyocyaneus* also S.B.F. scores the highest point in bacteriostasis, S.A.F. coming second, and S.T.F. third in the test. Sulphathiazole does not cause any bacteriostatic effect on this organism even at a concentration of 300 mg. per cent. Against *sh. dysenteriae* (Shiga) all the compounds are equally powerful, and exert bacteriostasis in very low concentration (12.5 mg. %). Tests with *Sh. sonnei* however show that S.T.F. does not possess any demonstrable effect even at a concentration of 100 mg. %, while S.A.F., S.B.F. and sulphathiazole at the same level give similar bacteriostatic effects. Against *Eberthella typhosa* also, all the compounds, are equally active, but with *S. paratyphi* A., S.A.F. and S.B.F. appear to be more bacteriostatic than Sulphathiazole and S.T.F., the latter being the least active compound, in this respect. (Table II).

From an analysis of all the data so far recorded, it seems plausible to infer that the more soluble formaldehyde condensation products of sulphonamides (S.A.F. and S.B.F.) appear to exert more powerful bacteriostatic activity than the insoluble condensation product such as S.T.F. It also seems likely that condensation with formaldehyde leading to the incorporation of the  $\text{CH}_2\text{OH.NH}$  group to the sulphonamide molecule has led to an increase in the potency as well as in the range of activity of sulpha drugs. It may be probable that such condensation has led to a change in the molecular properties in such a way that compounds liberate the active anionic form in the system making them resonant to play their part in the drug-enzyme competition. The lability of these compounds also ensures liberation of formaldehyde in the body such as in the genito-urinary tract, and thus helps in tackling the infections both at acid and alkaline ranges of pH. They are, therefore, likely to be of double advantage for therapeutic purposes.

Hence it may be suggested that the formo derivatives particularly, S.A.F. and S.B.F. should be given more extensive trials in the clinical field, against all sorts of infections to bring out their therapeutic possibilities.

#### SUMMARY

1. The formaldehyde condensation products of sulphathiazole (S.T.F.), sulphacetamide (S.A.F.), Sulphabenzamide (S.B.F.), have been screened against various pathogens, in comparison with sulphathiazole (S.T.).

2. Against *Streptococcus viridans*, S.A.F. and S.B.F. appear to be the best active bacteriostatic agents; S.T.F. and ST are inactive in this respect.

3. S.B.F. exerts the highest bacteriostatic action against the urinary pathogens, —*B. pyocyaneus* and *Staphylococcus aureus*.

4. Against *E. coli* and *B. proteus*, S.A.F. and S.B.F. exert similar activity. S.T.F. and S.T. are inactive against these two organisms even at the concentration of 200 mg.‰.

5. All the "formo" compounds as well as S.T. exert powerful bacteriostatic effect against *Sh. dysenteriae shiga* and moderately high effectiveness against *E. typhosa* (at 100 mg.‰).

6. Against *sh. sonnei* S.T.F. gives the lowest bacteriostatic effect, while the rest of the compounds produce the same activity.

7. It is suggested that "formo" derivatives, particularly S.A.F. and S.B.F. be given more extensive trials in the clinical field.

In conclusion, my thanks are due to Mr. Amal Das Gupta, B.Sc. for technical assistance in this work.

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## STUDIES ON THE NUTRITIVE VALUE OF GERMINATED SOYA-BEAN AND SOYA-MILK

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The digestibility and biological value of raw soyabean is not high and is almost the same as the other Indian pulses possess. This observation was made by Aykroyd, Ahmed, Patwardhan and Basu (1). The same observation was made by Osborne and Mendel (2), Vestal and Shrewsbury (3) and Vestal, Shrewsbury and Hauge (4). They showed that when rats were fed with raw powdered soya-bean, the diet being otherwise adequate and soyabean being the sole source of protein, normal growth was not possible. On the other hand Everson *et al* (5) made a comparative study of the nutritive value of soyabean in the raw state and after germination for 60 hrs. To one group of rats he gave raw soyabean in the powder form and the another group he gave the same soyabean after germination for 60 hrs, both the groups of rats being maintained on a diet of 10% protein level. The increase in weight per g. of protein fed observed by him was 0.48 in case of raw soyabean and 1.4 in case of the germinated bean.

Previous communications from this laboratory (De and Subrahmanyam, 6), Desikachar *et al*, (7) have shown that germination increases the nutritive value of soyabean milk. The present investigation was intended to study the effect of germination on the nutritive value of soyabean when fed in the form of powder and also when fed in the form of milk i.e. in emulsified form.

## EXPERIMENT

25 rats weighing between 40 to 50 g. were taken and these rats were divided into five groups each group consisting of 5 rats. The protein level was maintained at 10% in all the groups of rats and the source of protein was as follows in the five groups of rats.

First group—raw soyabean powder.

Second group—soyabean that was germinated for 48 hrs.

Third group—milk prepared out of soyabean that was allowed to germinate for 48 hrs.

Fourth group—Cow's milk brought from Dairy Research Institute, Bangalore, every day.

Fifth group—a mixture of the same cow's milk given to the 4th group and the same soyabean milk given to the 3rd group in the proportion of 20% and 80%.

The basal diet given to all the five batches was common and was prepared according to the following formula.

Salt mixture—4% Osborne and Mendel (8).

Cocconut oil—5%

Sugar—10%

Starch—81%

Yeast tablets—4 tablets of Squibbs Brewers Yeast per 100 g. of the diet.

Besides this each rat was given 2 drops of Shark liver oil per week and sufficient calciferol. The protein level of the diet was maintained in all the five groups at 10%.

The first and second groups of rats were given diet as follows:—Every day at about 10 A.M. the diet was weighed in cups and made into paste with water and given to the rats. The next morning at about 9 A.M. the diet cups were cleaned and the residue left each day was collected in separate bottles—one bottle being specified for one rat and at the end of one week the total collected refused diet was dried and weighed. From the difference between the weights of the total diet given per week and the weights of the collected refused diet the food intake was calculated.

The soyabean milk given to the 3rd group of rats was prepared as follows according to the process of De and Subrahmanyam (loc cit): Soyabean was allowed to germinate for 48 hrs., and then the skins were peeled off and the kernels were extracted with 5 times water containing 0.02% Sodium bicarbonate for 15 minutes at 75°C.

The kernels now obtained were pasted, mixed with 5 times water and strained through cloth and finally boiled.

For the fourth group of rats cow's milk was brought from the Dairy Research Institute, Bangalore, daily, boiled for a few minutes, cooled and then given.

The basal N. free diet, that was supplied to the last three groups of rats i.e. the rats that were provided with milk, was weighed in cups and made into paste with water. The milk was given in a separate cup. To maintain the protein level at 10% the supply of milk was adjusted with the average intake of N. free diet. Every day at about 9 A.M. the N. free diet was given to the rats and water was given in the milk cup. The milk was subsequently given at about 3 P.M. in the milk cup throwing away the water. Every day the protein content of soyabean milk and cow's milk was determined from an aliquot portion and the protein supplied calculated on weekly basis.

The preliminary period of experiment in all the five groups was one week and the experiment was conducted for five weeks. The rats were weighed at the end of each week. The following results were obtained.

Group No.	Source of protein.	Average			
		Grain in wt. in g.	Food intake in g.	Protein intake in g.	Grain in wt. per g. of protein
I.	Raw soyabean powder	26.5	277.3	27.73	0.95
II.	Soyabean germinated for 48 hours	31	233.0	23.30	1.32
III.	Milk from soyabean germinated for 48 hours	40.1	284.81	28.5	1.43
IV.	Cow's milk	39.2	272.75	27.20	1.44
V.	Mixture of 20% cow's milk and 80% Soyamilk	40.4	260.21	26.02	1.55



## DISCUSSION

From the figures in the Table, it will be evident that the nutritive value of soyabean increases a good deal on germination. It was interesting to observe that there was a considerable falling of hair in all the rats of both the groups receiving raw and germinated soyabean in the powder form. Johnson *et al* (9) showed that soyabean protein contains a sulphur-nitrogen containing complex which is absorbable to rats, but cannot be used for tissue building purposes and that on heating the soyabean it becomes available. Here the conclusion appears to be inescapable that germination cannot make the unavailable part of the sulphur containing amino acids available as heating and other process do and that the increase in the nutritive value is due to some other reason and not due to the liberation of S-amino acids.

The other interesting observation was that the rats that were given soyabean milk i.e. the rats of group 3 had quite healthy hair appearance. There was no falling of hair—on the contrary the hair of all the rats was glossy and had silky appearance. The milk was always boiled at the time of preparing. From the very appearance of the hair of the rats the conclusion seems to be that the S-amino acids present in the soya-milk are available. As the milk was boiled sufficiently so it cannot be definitely said whether they were available due to emulsification or due to heating. In the emulsified state the sulphur-nitrogen complex of the protein might be more vulnerable to the attack of the proteolytic enzyme. But it is more probable that heat plays the most important role here.

## SUMMARY

1. The effect of germination on the nutritive value of soyabean was studied by rat growth method and it was found that while raw soyabean powder gives a biological value of 0.95, after 48 hrs. germination the biological value of the soyabean powder becomes 1.33.

2. The effect of germination on the nutritive value of soyabean milk was studied and it was found that on 48 hrs. germination the biological value becomes 1.41 where as cow's milk (from Imperial Dairy Research Institute) gives 1.44.

3. The effect of supplementing milk prepared from germinated soyabean with 20% cow's milk was studied and it was observed that supplementing increases the biological value.

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## THE EFFECT OF CERTAIN SUBSTANCES ON THE BIOSYNTHESIS OF ASCORBIC ACID BY PHASEOLUS MUNGO DURING GERMINATION

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The increase in the ascorbic acid content of the pulses during the process of germination was reported by several workers (1-6). Ahamad and his associates (1) found that when *phaseolus mungo* was germinated with different sugars there was an increased synthesis of ascorbic acid. They also observed that minerals like manganese chloride, boric acid, cadmium chloride accelerate the synthesis of ascorbic acid. Chlorophyll, thiamine chloride and carotene gave no positive response. Ray (5) observed an increased synthesis of ascorbic acid in peas (*pisum sativum*) allowed to germinate with different hexoses, while pentoses were almost without any effect. Rudra (6) reported that manganese sulphate stimulates the production of ascorbic acid in germinating peas. King and his associates (7) observed that when rats were fed chloretone, camphor, menthol, geraneol, terpineol, aspirin etc. the animals excrete large amounts of ascorbic acid in urine. Roy (8) and his associates, however, reported that rats made deficient in thiamine and riboflavin when fed chloretone do not excrete increased amounts of ascorbic acid.

In the present paper, the studies on the effect of various antiseptics, hypnotics, analgesics and B-Vitamins on the increased production of ascorbic acid by *phaseolus mungo* during the process of germination have been presented.

## EXPERIMENTAL

*Germination of the seeds of Phaseolus Mungo:* 5 g. of clean and dry seeds of *phaseolus mungo* were embedded in a sterilized petri dish containing sterilized and purified sand. The seeds in each dish were soaked with 5 cc. of distilled water. After twenty four hours all the seeds swelled up, seed coats ruptured and hypocotyls came out. 5 cc. of a 0.1 per cent aqueous solution of the different chemicals, whose effect on the germination and on the ascorbic acids content of the seeds were studied, were added daily to different petri dishes till the experiment was completed on the sixth day of germination. 5 cc. of distilled water only were added daily to the control dishes. The petri dishes were kept away from the direct sunlight during the course of germination.

*Extraction of Ascorbic Acid of Phaseolus Mungo before and during the course of germination:* Ascorbic acid in *phaseolus mungo* was extracted as follows: the contents of the petri dish containing sand and seeds, germinated or ungerminated, were transferred to a glass mortar containing 25 cc. of a freshly prepared 3 per cent solution of metaphosphoric acid and ground under the acid with a glass pestle. The contents were then transferred to a measuring cylinder, citrate buffer was added to bring the pH to 3.5, the volume was made upto 100 cc. and filtered. The filtrate was used for the chemical estimation of ascorbic acid.

*Chemical estimation of Ascorbic Acid:* (a) *Free ascorbic acid:* An aliquot of the extract was titrated with a 0.02 per cent solution of 2,6 dichlorophenol indophenol standardised according to the method of Menaker and Guerrant (9) and the value for free ascorbic acid was calculated (b) *Dehydro-ascorbic acid:*—To an aliquot of the original extract, sulphuretted hydrogen was passed for ten minutes which converted dehydroascorbic acid into free ascorbic acid. Sulphuretted hydrogen was chased out by a current of carbon dioxide and extract was titrated against a standardised solution of 2,6 dichlorophenol indophenol. The result gave the value for the sum total of free ascorbic acid and dehydro-ascorbic acid. By deducting the value for free ascorbic acid the dehydroascorbic acid value was calculated. The result are given in Tables I and II. The effect of different chemicals on the growth of the seedlings were also noted.

*Biological Assay of Ascorbic Acid:* To study if the ascorbic acid values obtained by titration with the dye was actually ascorbic acid and not other non-specific reducing substances, ascorbic acid values of germinated pulses were determined biologically by the well known curative method of assay of guineapigs. Healthy male guineapigs of weights varying between 225 g. and 250 g. were fed a scorbutic diet (10) for one week. Every animal was fed 5 mg. of ascorbic acid per day and two drops of concentrate of vitamin A and D twice a week. The animals which grew normally were selected for the experiment and were fed only scorbutic diet. The animals were weighed on alternate days. When the animals began to lose weight from the seventeenth day of the experiment they were paired.

Germinated seeds were picked up from the sand bed, crushed in a glass mortar and the juice was extracted through a gauge cloth. Ascorbic acid in an aliquot of the juice was extracted with metaphosphoric acid and the total ascorbic acid value was determined.

TABLE I

*Ascorbic acid Content of Germinating 'Phaseolus Mungo' Treated with different B Vitamins.*

*(Ascorbic Acid values are Expressed in Mg. per cent).*

Substances used for germination.		Days of germination.								Remarks.
Water only		1		3		5		6		
	Free ascorbic acid	43.4	0.1	69.9	0.8	0		0		Standard growth.
	Dehydro ascorbic acid	13.6	0.9	32.9	0.8	120.0	0.8	74.1	0.9	
Nicotinic Acid	Free	42.1	1.0	77.4	1.2	36.4	1.0	0		Standard
	Dehydro	8.9	2.0	21.1	0.3	93.0	0.5	62.2	1.5	growth.
Choline	Free	54.7	1.1	100.2	1.1	0		0		Growth
chloride	Dehydro	7.5	0.5	11.9	0.1	136.8	2.0	100.6	1.6	Accelerated.
Calcium	Free	51.6	0.1	89.6	0.2	0		0		Growth
pantothenate	Dehydro	14.6	0.5	14.6	1.6	128.2	1.6	115.2	2.6	Accelerated.
Thiamine	Free	44.6	0.6	70.9	0.8	0		0		Standard
hydrochloride	Dehydro	9.6	0.2	22.7	0.5	110.2	1.9	112.6	2.2	growth
Para-amino	Free	56.2	0.7	78.8	1.1	23.2	0.8	0		Growth
benzoic Acid	Dehydro	12.0	0.3	11.1	0.1	85.1	0.7	112.6	1.8	slightly retarded.
Inositol	Free	50.2	1.0	83.3	1.2	23.2	0.8	0		Growth
	Dehydro	9.7	0.3	18.3	0.9	85.1	0.7	103.4	1.1	Accelerated
Pyridoxine	Free	58.2	0.8	96.2	1.2	57.1	1.8	0		Growth
hydrochloride	Dehydro	11.5	0.3	10.6	1.2	46.1	0.1	106.8	1.1	Accelerated
Nicotinamide	Free	51.9	0.8	89.2	1.1	22.8	0.1	0		Standard
	Dehydro	7.6	0.1	11.1	0.3	70.1	1.0	101.6	1.8	growth

Before germination the ascorbic acid value of *phaseolus mungo* was 12.6 ± 0.1. The seeds did not contain dehydroascorbic acid.

TABLE II

*Ascorbic acid content of Germinating 'Phaseolus Mungo' Treated with different Antiseptics, Analgesics and Hypotics.*

*(Ascorbic Acid values are expressed in mg. per cent).*

Substances used for germination.		Days of germination.								Remarks.
Water		1		3		5		6		
	Free ascorbic acid	49.5	0.9	70.8	1.2	0		0		Standard growth.
	Dehydro ascorbic acid	8.5	0.2	34.8	0.3	125.6	1.2	82.6	1.8	
Acetone	Free	52.2	0.6	96.4	0.4	0		0		Standard
	Dehydro	11.1	0.6	14.1	1.4	138.1	1.2	99.2	1.0	growth.
Terpeneol	Free	39.1	0.5	97.2	1.1	25.5	1.3	0		Growth
	Dehydro	13.2	0.6	12.2	0.5	111.3	0.8	96.4	1.0	retarded.
Geraneol	Free	38.9	0.3	98.6	0.4	16.1	0.1	0		Growth
	Dehydro	17.3	0.6	26.0	0.6	113.9	2.1	110.2	1.2	retarded.
Menthol	Free	48.4	2.0	97.0	1.6	8.1	0.1	0		Growth
	Dehydro	10.2	0.2	8.2	0.4	122.0	1.0	100.2	1.2	retarded.
Camphor	Free	48.1	0.1	91.6	1.9	7.7	0.2	0		Growth
	Dehydro	10.4	1.4	16.1	0.3	127.0	1.5	73.6	1.1	retarded.
Thymol	Free	38.1	1.3	6.2	0.7	Seedlings were dead due to toxic action.				
	Dehydro									

One animal of each pair was fed an aliquot of the pressed juice containing 5 mg. of crystalline ascorbic acid dissolved in 1 cc. of water. The rates of growth of animals in each pair were comparable which indicated that the ascorbic acid in the germinated seeds is true ascorbic acid and not other non-specific substances.

## RESULTS

Of the B-vitamins choline chloride, pyridoxine hydrochloride, calcium pantothenate and inositol accelerated the growth of the seedling and enhanced the production of free ascorbic acid during the course of germination as compared to the controls germinated with water only. This rise in the production of the ascorbic acid was maximum on the third day of germination. On the fifth or sixth day of the germination the free ascorbic acid value became nil but there was a considerable rise in the dehydroascorbic acid value. The growth of the seedlings was not enhanced with either nicotinic acid or nicotinamide but there was increased production of ascorbic acid upto the third day of germination. Para-aminobenzoic acid although enhanced the production of ascorbic acid slightly retarded the growth of seedling. The effect of thiamine chloride was similar to that of water alone.

Acetone, terpineol, geraneol, menthol, camphor, veronal, luminal and chloretone accelerated the production of free ascorbic acid which was maximum on the third day of germination and disappeared on the fifth or sixth day of germination. Terpeneol, geraneol, menthol, camphor and chloretone slightly retarded while veronal and luminal stimulated the growth of the seedlings. With acetone the growth of the seedling was normal. Thymol had a toxic action on the seedlings which did not grow at all.

In all the cases dehydroascorbic acid value, which was absent in dry seeds went on increasing and was maximum either on the fifth or on the sixth day of germination when the free ascorbic acid value became absent.

## DISCUSSION

The embryo remains dormant in the dry-pulses and very little ascorbic acid is present in them. During the process of germination increased amounts of ascorbic acid appear in the seeds. Substances like cholinechloride, pyridoxinehydrochloride, calcium pantothenate, inositol, nicotinic acid, nicotinamide, chloretone, menthol-camphor, geraneol, terpineol, acetone, veronal, luminal etc. produce a further increase in the ascorbic acid content of the germinating *Phaseolus mungo*. 24-hours after the seeds are soaked with water the seed coats rupture and hypocotyls come out so that all the substances came in direct contact of the seedlings to produce their effect on germination and on the ascorbic acid content of the seeds. The free ascorbic acid content of the seedling was maximum on the third day of germination and disappeared completely either on the fifth or the sixth day of germination. The abrupt fall in the free ascorbic acid content seems to be due to the formation of dehydroascorbic acid.

The dry seeds did not contain any dehydroascorbic acid which went on rising during the course of germination and the value became maximum on the fifth or sixth day of germination when the free ascorbic acid value became nil. The formation of the dehydroascorbic acid during the process of germination may be either due to the presence of the oxidative enzymes like ascorbic acid oxidase present in the germinating pulses which convert ascorbic acid into dehydroascorbic acid or due to the rapid oxidative metabolism in the germinating seeds.

Owing to the great dissimilarity of the nature of the different compounds found to increase the production ascorbic acid during the course of germination it seems that the substances stimulate the increased synthesis of ascorbic acid by the germinating seedlings and do not act as precursors of ascorbic acid.

#### SUMMARY

1. The effect of some of the vitamins of the B-group, antiseptics and hypnotics on the increased production of ascorbic acid in *phaseolus mungo* during the process of germination has been studied.

2. Choline chloride, pyridoxine hydrochloride, calcium pantothenate, inositol, nicotinic acid, nicotinamide and para-aminobenzoic acid have been found to accelerate the production of free ascorbic acid in the germinating seedling upto the third day of the germination. Thiamine chloride has no extra effect on the synthesis of ascorbic acid.

3. Aceone, terpenecol, geraneol, menthol camphor, veronal, luminal and chloretone also enhanced the synthesis of ascorbic acid by the germinating seedlings.

4. Dehydroascorbic acid which is absent in dry seeds of *phaseolus mungo* was formed and became maximum on the fifth or sixth day of germination when the free ascorbic acid completely disappears.

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## CATALYTIC EFFECT OF TITANOUS AND STANNOUS CHLORIDES ON THE ACID HYDROLYSIS OF CASEIN

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Sullivan and Hess (1) reported that use of small amounts of titanium chloride in the hydrolysis of proteins with hydrochloric acid resulted in a reduction in humin formation and a more rapid liberation of cystine. Lieben (2) studied the hydrolysis of casein, gelatine and Bacto peptone by sulfuric acid under different experimental conditions and using a variety of metallic salts. He found that titanous chloride as well as stannous and stannic chlorides increased the rate of hydrolysis to an appreciable extent, while copper, nickel and manganese salts were without effect.

The present report deals with studies on the catalytic effect of titanous chloride and stannous chloride on the hydrolysis of casein by varying concentrations of sulfuric and hydrochloric acids and on the tryptophane and cystine contents of the resulting hydrolysates.

### EXPERIMENTAL

*Materials used:*—Commercial Argentine casein (N content 12.0 per cent.) was used for all studies reported here. Solid stannous chloride (C.P.) and 15 per cent. solution of titanous chloride were the catalysts employed.

In all experiments in which stannous chloride was the catalyst, the casein concentration was 1 g. per 20 cc. of acid (*i.e.* 5 per cent.). The use of a 15 per cent. solution of titanous chloride as catalyst, however, resulted in a slightly lower concentration of casein as well as the acid; an equal volume of water was added for compensation to the control flask without the catalyst.

The hydrolysis was carried out in 250 cc. Erlenmeyer flasks fitted with reflux condensers and placed in an oil bath maintained at a temperature of 120-125°C. at which the mixture was kept gently boiling. A few pieces of pumice were added to aid boiling and a few drops of caprylic alcohol to prevent frothing in the earlier stages. Samples were drawn at regular intervals to study the extent of hydrolysis and for determination of the tryptophane and cystine contents of the hydrolysates. The samples were immediately cooled and neutralised with 10 per cent. sodium hydroxide added dropwise to a faintly acid reaction to litmus (pH approximately 6.0).

The titanium was almost completely precipitated as hydroxide in this neutralisation and was removed by filtration, the precipitate being washed repeatedly with hot water. During the first three experiments, it was observed that a small amount of titanium still remained in solution and caused a turbidity in the subsequent colorimetric determination of cystine. In later experiments, therefore, the sample was made definitely alkaline and sodium carbonate was added till there was no further precipitation and the titanium-free filtrate was neutralised with acid.

Tin was removed completely as sulfide by passing hydrogen sulfide through the slightly acid and warm sample, filtering and washing the precipitate with hot water. Complete precipitation was tested for by further dilution and passing  $H_2S$ . The filtrate and washings were combined and boiled to remove the excess of hydrogen sulfide.

#### ANALYSES

The titanium or tin-free samples were used for all determinations. Total nitrogen was determined by Kjeldahl method and *alpha*-amino nitrogen by the Van Slyke nitrous acid method. When casein was hydrolysed with 6N  $H_2SO_4$ , the maximum value for amino N/Total N was found to be 81 per cent. and this was, therefore, designated as 100 per cent. hydrolysis, the extent of hydrolysis in each case being calculated and reported on this basis. Cystine and tryptophane were estimated by Block and Bolling's modifications of the Winterstein-Folin and Millon-Folin procedures, respectively; these methods were recommended by Block and Bolling (3) as accurate. In order to liberate all the tryptophane, the samples were subjected to a further alkaline hydrolysis by adding solid sodium hydroxide to 5N strength and heating in oil bath with refluxing for about 5 hours. Cystine gets converted into cysteine in presence of titanium and tin and hence, for determination as cystine, the samples were acidified with hydrochloric acid, concentrated to a syrupy consistency on steam bath with frequent stirring and then again diluted to original volume by adding water after decolorisation with active carbon. A Klett-Summerson photo-electric colorimeter was used for color comparisons with filter S 42 for tryptophane and S 54 for cystine. The values are expressed in milligrams per 16 grams of nitrogen.

The results are presented in the accompanying table.

#### DISCUSSION

Catalytic Effect of Titanous and Stannous Chlorides on the Acid Hydrolysis of Casein.

Expt. No.	Acid conc.	Catalyst.	Period of hydrolysis in hours.	Hydrolysis.			Tryptophane, mg./16g.N.	
				without catalyst. (a)	with catalyst. (b)	catalytic effect. (b-a)%* (a)	without catalyst.	with catalyst.
1	H <sub>2</sub> SO <sub>4</sub>	20%		66.7	66.0	- 1.1	—	—
2	"	"	4	62.7	67.6	+ 7.8	443	534
3	"	1.125%	3	71.2	77.8	+ 9.3	389	664
4	"	"	4	55.0	58.3	+ 6.0	503	378
5	"	1.5%	5	73.2	72.6	- 0.8	375	770
6	"	"	2	49.8	51.3	+ 3.0	725	635
7	"	"	1	52.4	59.7	+ 13.9	525	555
8	"	2.25%	2	66.7	69.7	+ 4.5	405	583
9	"	"	4	62.5	67.0	+ 7.2	358	278
10	"	"	1	76.3	78.0	+ 2.2	234	75
11	"	"	2	84.8	82.3	- 2.9	148	589
12	"	"	2	61.1	66.7	+ 9.2	423	404
13	"	"	4	76.8	83.3	+ 8.5	322	174
14	"	1.5%	1	89.2	92.7	+ 3.9	119	1126
15	"	"	2	45.4	58.4	+ 28.6	902	591
16	"	"	4	59.5	67.4	+ 13.2	438	1127
17	"	"	1	48.4	55.0	+ 13.6	869	565
18	"	2.25%	0.5	63.2	67.4	+ 6.6	402	39
19	"	"	1	97.5	96.4	- 1.1	Trace	Trace
20	"	1.5%	6	99.1	98.5	- 0.6	1122	1385
21	"	"	8	31.9	35.0	+ 9.7	963	1241
22	"	"	1	41.1	41.2	+ 0.2	831	947
23	"	"	2	51.7	49.6	- 4.1	1090	1311
24	"	"	4	31.8	33.9	+ 6.6	865	1119
25	"	"	0.5	39.0	40.7	+ 4.3	904	904
26	"	"	1	51.0	50.1	- 1.8	731	731
27	"	"	2	68.0	65.2	- 4.2	551	613
28	"	"	1				287	385
29	"	"					152	372
30	"	"					259	392
31	"	"					222	382
32	"	"					205	370
33	"	"					159	248
34	"	"					231	281
35	"	"					202	251
36	"	"					147	166

\* Probable acceleration

prob. for acceleration

Casein concentration was 5 per cent. in all experiments.  
Temperature of hydrolysis 120-125°C.  
Tryptophane content of the Argentine casein was 1565 g./16 g. N.



In experiments 9 and 10, using titanous chloride and tin chloride, respectively, as catalyst and 14 per cent. hydrochloric acid as hydrolytic agent, additional samples were drawn after half an hour of hydrolysis and the results confirmed the finding that catalytic effect was much higher during initial stages of the hydrolysis. In experiments 11 and 12, the hydrochloric acid concentration was reduced to 1N and 2N, but no greater catalytic effect was seen at these low concentrations of acid.

These results confirm the findings of Lieben (2) that both titanous chloride and stannous chloride accelerate the hydrolysis of proteins by acids. His results, when calculated on similar basis, showed a decrease in catalytic effect during later stages of hydrolysis, but he did not notice this trend. It is also to be noted that the catalytic effect was never as great as the figures given by Lieben would indicate.

The tryptophane content of the catalyst-treated hydrolysate was generally higher than that of the control, indicating that both titanium chloride and stannous chloride exert a slight protective action on tryptophane during acid hydrolysis of proteins.

A few cystine determinations made on the hydrolysates showed that both the catalysts liberated cystine early as was observed by Sullivan and Hess (1) and had, besides, a protective action on it. Loss of cystine is reported to be 20-35 per cent. during acid hydrolysis under varying conditions [McFarlane *et al.* (4), Pollard and Chibnall (5), Miller and du Vigneaud (6), Bailey (7), Sullivan *et al.* (8), Hess *et al.* (9)]. The losses of cystine observed here, particularly in the controls were somewhat greater and this is probably attributable to the presence of impurities in the commercial casein used ; these are known to increase cystine destruction in acid hydrolysates (Lugg, 10).

#### SUMMARY

The catalytic effects of titanous chloride and stannous chloride on the hydrolysis of casein by sulfuric and hydrochloric acids have been studied.

The catalytic effect was marked during the initial stages of hydrolysis. After about four hours of boiling, no catalytic effect was observed ; on further heating, there was a slight retardation in the rate of hydrolysis in presence of the catalysts.

Both catalysts were found to exert a slight but definite protective action on tryptophane and cystine during acid hydrolysis of proteins.

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EFFECT OF THE PROTEOLYTIC INHIBITOR ON THE BIOLOGICAL  
VALUES AND SUPPLEMENTARY VALUES OF DIFFERENT VARIETIES  
OF SOYA BEAN

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It has been concluded by the Soya-bean Sub-Committee (1), that Soya bean in spite of being a superior pulse as compared with others in its net protein value, fat, minerals and vitamin contents, did not prove itself a better supplement to a poor rice diet. This was accorded to the presence of a proteolytic inhibition factor present in the soya-beans by Bowman (2). Experiments have been conducted by Ham *et al* (3, 4) and De *et al* (5) by feeding to animals an acid extracts of the bean (which extracts this factor), as well as the isolated protein, wherein a positive evidence of inhibition in growth has been observed.

The factor which mainly attracted our attention, to carry out this work was the wide variation in the biological values of the different varieties of soya beans ranging from 44 to 76 (1). This wide variation was presumed, to be due to the presence of the proteolytic inhibition factor, which as reported by Shastery *et al* (6) was present in varying amounts in different varieties of soya beans. Further, it was found that no data was available about the degree of inhibition along with the biological value and the supplementary value of different varieties of soya beans grown under identical conditions.

In order to bring the points, which were so far divergent, to a clear range, 30 different varieties of soya beans were obtained from the Government Agricultural Farm, Hebbal Bangalore, which were grown on their experimental farm, under identical conditions.

The plan of experiment was to determine the degree of inhibition of all the varieties of soyabeans and select a few varieties showing maximum and minimum inhibition. These selected ones were used for the determination of their biological and supplementary values.

## EXPERIMENTAL

### *Degree of inhibition:*

(1) *Isolation of the inhibitor.* The beans were finely powdered to pass through 29 mesh and 2.5 g. of the powder was shaken in a stoppered bottle for 3 hrs. with 50 c.c. of dilute  $H_2SO_4$  so that reaction of the solution was  $pH 4.2$ . The solution was then kept overnight in a refrigerator, and the next morning it was filtered through a thin cloth. The filtrate was centrifuged and the supernatant liquid collected.

The liquid contained the inhibitor which was always used fresh.

(2) *Preparation of the enzyme:* 20 g. of pancreas was taken in 100 c.c. 20 per cent glycerine, shaken well and kept overnight in a refrigerator. It was then filtered through a fine cloth.

Having thus prepared the inhibitor and the enzyme, fresh cow's milk was taken, boiled and its  $pH$  adjusted to 7.0 with dilute  $NaOH$ . The  $pH$  of the inhibitor was also adjusted likewise to 7.0. 50 cc. of milk was taken and 1 cc. of the prepared inhibitor and 1 c.c. of the enzyme solution were added and incubated at  $37^\circ C$  for 3 hours. A control (containing only the enzyme and not the inhibitor solution) was carried along-with. After the incubation an aliquot of the partly digested milk was taken to which an equal volume of 10 per cent trichloroacetic acid solution was added to precipitate the undigested proteins. It was then filtered and the filtrate was digested for the estimation of its nitrogen content. All the varieties of soya beans were thus examined and the results tabulated as follows, on the basis of 100 per cent digestion in the control.

TABLE I

No.	Name of variety.	Degree of inhibition.	No.	Name of variety.	Degree of inhibition.
15.	S.B. Yellow No. 1	29.93	16.	S.B. White mammoth	30.70
17.	S.B. 4	30.00	18.	S.B. Yellow No. 1	29.93
19.	S.B. 13 A	29.88	20.	S.B. 10	29.87
21.	S.B. Cac. 2	29.28	22.	Shillong Black No. 1	28.30
23.	S.B. 16	27.92	24.	Bihar White	27.92
25.	S.B. Big	26.82	26.	White soyabean	26.41
27.	S.B. 13 B	26.24	28.	S.B. Calc. 7	25.93
29.	S.B. 2 Black	25.77	30.	S.B. Pyneagree	24.02

Amongst the above thirty varieties four were chosen which showed maximum and minimum inhibition, for the determination of their supplementary and Biological values.

The varieties chosen were the following:—

S.B. 3 Black	...	39.03	Maximum inhibition.
S.B. Yellow	...	38.96	
S.B. 2 Black	...	24.25	Minimum inhibition.
S.B. Pyneagree	...	24.02	

*Supplementary value to South Indian diet.*

18 rats weighing between 65-75 g. were selected and divided into three groups of six in each, so that the rats in the three groups compared with one another with respect to weight, litter, sex etc. Group I served as the control and received the basal diet, group II received the basal diet with 10 per cent of the variety of soya bean containing the maximum amount of inhibitor and group III received the basal diet with 10 per cent of the variety of soya bean containing the minimum amount of inhibitor. The composition of the basal diet used is as follows:—

1. Rice (polished)	...	...	80 parts
2. Thur dhal	..	...	50 "
3. Brinjals	...	..	80 "
4. Leafy vegetables	..	...	24 "
5. Common salt	..	...	0.3 "
6. Oil (gingely)	...	..	7 "

The basal diet without the vegetables was prepared every week but the vegetables and the soyabeans powder were added daily to the diet. The rats were weighed every week and a record of their food consumption was maintained. The experiment was continued for five weeks and the results tabulated as follows.

TABLE II

*Supplementary values to poor rice diet.*

Group.	Rat No. and sex.		Initial weight in g.	Food consumed in g.	Increase in weight in 5 week in g.	Average weekly increase of wt. in g.
Basal diet.	(1)	1. M	69.0	350	14	2.6
		2. M	62.0	358	26	5.1
		3. M	70.0	364	22	4.3
		4. F	72.0	362	21	4.2
		5. F	69.0	352	17	3.4
		6. F	72.0	355	11	2.3
	Average					3.65
Basal diet.	(2)	1. M	75.0	373	43	8.5
		2. M	69.0	378	28	5.6
		3. M	70.0	364	19	3.8
		4. F	67.0	352	20	4.2
		5. F	72.0	357	15	2.9
		6. F	72.0	387	46	9.2
	Average					5.70
Basal diet.	(3)	1. M	72.0	371	30	5.9
		2. M	70.0	358	18	5.6
		3. M	65.0	371	28	5.5
		4. F	67.0	352	16	3.1
		5. F	69.0	375	24	4.8
		6. F	72.0	360	22	4.4
	Average					4.90

It is observed from the results that the group two getting a supplement of S. B. Pyneagre, whose degree of inhibition was 24.02, fared much better than the third group getting a supplement of S.B.3 Black with a degree of inhibition 20.05.

TABLE III

*Increase in weight with S.B. Yellow and S.B.2 Black as Supplements.*

Group.	Rat No. and sex.		Initial weight in g.	Food consumed in g.	Increase of wt. in six weeks in g.	Average weekly increase in wt. in g.
(1) Basal diet.	1.	M	46.5	434	11	1.8
	2.	M	51.0	467	42	7.0
	3.	M	46.5	454	35	5.8
	4.	F	49.0	443	26	4.3
	5.	F	51.0	450	29	4.8
	6.	F	51.0	455	32	5.3
Average						4.8
(2) Basal diet. + 10% S.B. Yellow	1.	M	48.0	471	48	8.0
	2.	M	52.0	467	40	6.7
	3.	F	52.0	458	40	6.7
	4.	F	47.5	460	47	7.8
	5.	F	50.0	414	43	7.1
Average						7.3
(3) Basal diet + 10% S.B. 2. Black.	1.	M	47.5	447	41	6.8
	2.	M	50.0	459	46	7.7
	3.	M	48.5	435	41	6.8
	4.	F	51.0	480	58	9.7
	5.	F	51.0	472	51	8.5
Average						7.9

Experiments with the S.B. Yellow and S.B.2 Black varieties show that the increase of weight due to the supplementary effect of these two varieties on the poor South Indian diet is 2.5 with the S.B. Yellow and 3.1 with the S.B.2. Black. Thus it was found out that the S.B. Yellow variety (degree of inhibition 36.96) showed less supplementary effect to the South Indian diet as compared with S.B.2. Black (degree of inhibition 24.25.)

The foregoing results show that the proteolytic inhibitor content of the soyabean has a definite role in the supplementary values of the soyabeans, and the presence of a higher amount of the inhibitor may be one of the factors responsible for a low supplementary value of the bean.

#### BIOLOGICAL VALUE

The biological value of two varieties of the soya beans showing maximum and minimum inhibition were determined by the Balance sheet method, the one recommended by Mitchell (1924).

Six rats weighing between 120—150 g. were used for the experiment. The rats were fed on a laboratory stock diet for one week and then fed on the nitrogen free diet consisting of:—

Starch	...	74.0	parts
Sugar	...	9.0	"
Oil	...	10.0	"
Salt mixture	...	5.0	"
Yeast extract	...	2.0	"

Adexolin was added to the feed twice a week at the rate of two drops per rat to provide vitamin A and D. The rats were kept on this diet for three days and from the fourth day their urine and faeces were collected for four days. After the expiry of four days the rats were given stock diet for four days and then given a diet containing soya bean having a protein level of 10 per cent.

No urine and faeces were collected for the first three days, as in the former case and the rest of the experiment was conducted as before. Similar collections of the urine and faeces were obtained by using another variety of soya bean. Food consumption records were maintained throughout the experimental period.

The urine and faeces were collected in special containers having preservatives. The preservative used for urine consisted of 5%  $H_2SO_4$  containing 20% phenol and 1% thymol. The faeces was collected daily and kept in the refrigerator. After the experimental period was over the collections of the urine and faeces were estimated for their nitrogen content.

The following tables give the figures of the two varieties of soya beans showing maximum and minimum inhibition content.

TABLE IV &amp; V

*Two varieties of soya beans showing maximum and minimum inhibition content.*

S. B. PYNEAGRE Group:—

	Weight of food nitrogen.	Weight of faecal nitrogen.	Weight of metabolic nitrogen.	Weight of urinary nitrogen	Weight of endogenous nitrogen.	Biological value.
1.	0.48573	0.16142	0.04688	0.09175	0.05495	82.633
2.	0.51230	0.17049	0.04358	0.10975	0.05530	78.143
3.	0.51621	0.17871	0.03067	0.26900	0.08050	45.39 <sup>8*</sup>
4.	0.51108	0.21423	0.03814	0.10825	0.04060	72.625
5.	0.50494	0.16610	0.04366	0.11205	0.07420	82.224
6.	0.53013	0.21045	0.03748	0.11350	0.07945	73.91 <sup>8</sup>
* (this animal was ailing)					Average	77.910

The results for the S. B. Pyneagre and S.B3. Black groups show that the Biological values of the two varieties vary according to their inhibitor content.

Variety	Degree of inhibition	Biological value
S.B. Pyneagre.	24.02	77.91
S.B. Black	39.03	60.80

### DISCUSSION

The comparative results for degree of inhibition, supplementary and Biological value are tabulated here to show the inter-relationship between them.

Variety	Degree of inhibition.	Supplementary value.	Biological value.
S.B. Pyneagre	24.02	5.70	77.91
S.B.3 Black	39.03	1.90	60.80
S.B. Yellow	38.96	7.30	—
S.B.2 Black	25.77	7.90	—

Those varieties of soya beans possessing higher concentrations of inhibitor showed that their supplementary and biological values were lower. There was a gain of 0.8 g. and 0.6 g. in the average weekly increase in weight with the varieties S.B. Pyneagre and S.B2. Black as against S.B3. Black and S.B. Yellow respectively. Further the biological value of S.B. Pyneagre was higher by 17.11 with respect to S.B3. Black. These results show that the concentration of inhibitor contained in a variety of soya bean has a definite role to play in its nutritive value, which shows itself up with the variation in the supplementary and biological values, and thus it can be said that the concentration of inhibitor of a variety of soya bean is one of the factors responsible for the wide variation of the supplementary and biological values of Soya beans.

In view of the above, it is recommended that those varieties of soya beans having a lesser degree of inhibitor should be selected and recommended for cultivation and use in order to achieve a higher nutritional return.

### SUMMARY

1. The degree of inhibition of thirty varieties of soya beans, which were grown under identical conditions, has been determined.
2. Supplementary value of four varieties of soya beans showing maximum and minimum inhibition have been determined.
3. Biological values of two varieties of soya beans showing maximum and minimum inhibition have been determined.
4. The supplementary and biological values of any variety of soya bean is higher when its degree of inhibition is lower.
5. The wide difference in the supplementary and the biological values of soya beans is due to the varying amounts of inhibitor they contain.



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## THE HEMATOPOIETIC PROPERTY OF THE IRON IN SOYABEAN AND SOYAMILK AND ITS UTILIZATION IN NORMAL RATS

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During the last few years the bioassay technic has been used extensively to determine the hematopoietic properties of various foods. The general plan has been to render the rats anemic by prolonged feeding of an exclusively milk diet and measure the increase in the level of haemoglobin at definite intervals after

administering different quantities of the experimental diet. Recent experiments have shown that the hematopoietic properties of different foods vary widely. The experiments of Elvehjem and Hart (1) have shown that the ionizable or non-hematin iron alone is the available iron in food. The available iron in this food can be determined by the colorimetric method of Elvehjem and Hart (*loc cit*).

However Smith and Otis (2) recently found discrepancies between the values for the ionizable iron in certain foods and the available iron. Mitchell, Myres, and Beard (3) have shown that the presence of copper and manganese are essential for the synthesis of haemoglobin. That a diet, consisting of whole milk, iron, copper and manganese is complete, was demonstrated by Kemmerev *et al* (4).

The available literature presents conflicting results regarding the relation-ship of ionizable iron and available iron. Most of the information regarding utilizable iron has been obtained by feeding experiments on rats rendered anemic by keeping on an exclusive milk diet. Under such abnormal conditions it is not unlikely, as pointed out by Pye and Mcleod (5) that factors not present in normal rats might give rise to complications. In present experiment, the technic of Pye and Mcleod (*loc cit*) which takes advantage of this has been employed. They perform the experiments on normal young rats following the haemoglobin values and determining the iron stored by them after an experimental period of six weeks.

The present work was undertaken to study the hamatopoietic properties of autoclaved soyabean and processed soyamilk. This question arises in view of the work of Desikachar *et al.* (6), which showed that soyamilk has a high nutritive value comparable to cow's milk. However since cow's milk is deficient in iron, the hematopoietic properties if any of soyamilk and soyabean powder needs investigation. Since raw soyabean contains an inhibitor which reduces the biological value of the proteins in it markedly, the soyabean powder obtained by grinding in an agate mortar was autoclaved for 30 min at 15 lbs pressure to destroy the inhibitor.

## EXPERIMENTAL

The total iron in whole milk powder and autoclaved soyabean was determined by Jarrow's modification of Kennedy's procedure (7). Whole milk powder and autoclaved soyabean contained respectively 7 mg. and 27.5 mg. of iron per 100 g. of substance.

# THE HEMATOPOIETIC PROPERTY OF THE IRON

Twentyfour male rats after weaning were placed in separate galvanized cages freshly treated with aluminium paint. The cages had raised bottoms of 5 mesh to prevent accumulation of faeces. The initial weights of the rats ranged from 30 to 45 g. Several littermates of the above were killed at start to determine the average iron content at the beginning of the experimental period. It is presumed that the initial iron content of the experimental rats is the same as their littermates which were analysed at the beginning.

The rats were divided into eight groups each comprising three. The initial haemoglobin level was found to range from 7.5 to 8.5 g. per 100 cc. of blood. This was determined by the Newcomer method with freely flowing blood obtained by cutting the tip of the tail. The groups were fed with the following diets.

TABLE I

Group No.	Diet Fed.
Group I.	
Group II.	Whole milk powder + .001 mg iron per gm body wt as autoclaved Soyabean
Group III.	Whole milk powder + .002 mg
Group IV.	Whole milk powder + .003 mg.
Group V.	Whole milk powder + .004 mg
Group VI.	Whole milk powder + 2.5 c.c. of soyamilk per 100 gm body weight
Group VII.	Whole milk powder + 5 c.c.
Group VIII.	Whole milk powder + Fe Cl. (.002 mg. iron per gm body weight)

One gram of milk powder was mixed with autoclaved soyabean powder containing the quantity of iron mentioned above for groups II to V. In case of groups VI and VII one gram of milk powder was mixed with the amount of soyamilk stated above. In group VIII, a small amount of milk powder was mixed with FeCl<sub>3</sub>. After consumption of the supplement 12 g. of milk powder were placed in the cups and the residue left on the next day was weighed. At the beginning of every week the rats were weighed and the amount of iron to be fed to each in the form of autoclaved soyabean powder, Soyamilk and Ferric chloride diet was varied accordingly. 1 cc. of a solution containing 0.5 mg. of iron per 100 cc. of diet was varied accordingly.

sulphate and .04 mg. of manganese as manganous chloride was added to supplement diet every day. The amounts of copper and manganese sufficient according to Rose and King (10). The level of hæmoglobin was determined by the Newcomer method every week in the same day as the weight was taken.

The table II. gives the amount of milk powder consumed by the rats during the experimental period of six weeks and the iron present in the milk powder consumed by it.

TABLE II

Group No.	Rat. No.	diet consumed. Amount of	Iron present in the milk powder alone.
I.	1.	... 380 g.	2.660 mg.
	2.	... 360	2.520
	3.	... 368	2.576
II.	4.	... 352	2.464
	5.	... 395	2.765
	6.	... 365	2.555
III.	7.	... 370	2.590
	8.	... 380	2.660
	9.	... 382	2.674
IV.	10.	... 330	2.310
	11.	... 390	2.730
	12.	.. 385	2.695
V.	13.	.. 388	2.660
	14.	. 388	2.716
	15.	. 400	2.800
VI	16.	.. 405	2.835
	17.	... 368	2.576
	18.	355	2.485
VII	19.	352	2.674
	20.	. 394	2.758
	21.	400	2.800
VIII	22.	.. 365	2.755
	23.	400	2.800
	24.	400	2.856

Table III. gives the number of grams of hæmoglobin per 100 cc. in each rat and the total increase in hæmoglobin level.

TABLE III

Rat No.	Initial level Hb in mg	Final level Hb in mg	Initial weight.	Final weight.	Inc. in weight.	Tot. inc. in Hb.	Inc. in Hb/g. in B. wt.
1.	8.1 g.	4.0	32 g.	95	63	-4.1	-0.0650
2.	7.7	4.2	30	88	58	-3.5	-0.0490
3.	7.4	4.0	33	102	69	-3.4	-0.0490
4.	7.7	6.9	35	105	70	-.8	-0.011
5.	7.9	6.8	34	108	74	-1.1	-0.014
6.	8.2	6.9	37	115	78	-1.3	-0.016
7.	8.3	11.0	38	125	87	2.7	0.031
8.	7.8	11.0	32	122	90	3.2	0.035
9.	7.2	10.8	34	119	85	3.6	0.043
10.	7.5	12.8	33	128	95	5.3	0.0558
11.	7.7	13.0	31	125	94	5.3	0.0560
12.	7.9	13.0	32	122	90	5.1	0.0567
13.	8.2	14.5	40	130	90	6.3	0.070
14.	8.0	14.3	35	120	85	6.3	0.078
15.	7.7	14.3	33	115	82	6.6	0.080
16.	7.8	11.0	37	130	93	3.2	0.0344
17.	7.9	11.3	38	132	94	3.4	0.0363
18.	8.1	10.8	36	125	89	2.7	0.0304
19.	8.3	14.2	36	128	92	5.9	0.0641
20.	7.0	13.3	40	121	81	6.3	0.0777
21.	7.7	13.1	33	110	77	5.4	0.0701
22.	7.8	14.1	33	124	91	6.3	0.0603
23.	8.1	14.6	40	128	88	6.5	0.0738
24.	7.3	13.9	37	125	88	6.6	0.0750

## DISCUSSION

The rats in group I were kept on an exclusive milk diet had a hæmoglobin level of 4% at the end. This is an agreement with the results of several workers. Rats fed with autoclaved soyabean sufficient to provide .001 mg./gm. body weight showed a slight reduction in hæmoglobin. The response to the iron containing supplement rapidly increases, with the amount of iron present in the diet until a level of .004 mg. of iron per gm. body weight in the form of soyabean powder produces slightly higher increase in the hæmoglobin level as obtained by feeding ferric chloride sufficient to provide .002 mg./gm. body weight. The ionizable iron present in the autoclaved soyabean is 56% of the total iron. Hence the addition of .004 mg. of total iron as soyabean is equivalent to the inclusion of .0022 mg. of ionizable iron. This produced slightly higher hæmoglobin response in the form of ferric chloride which is wholly ionizable i.e. about 60% of ionizable iron in autoclaved soyabean is utilisable for hæmoglobin regeneration. The iron from soyamilk is utilized to the same extent as that in ferric chloride.

Spinach, lettuce etc., though rich in iron are reported to be utilized very sparingly in rat. However the iron of the autoclaved soyabean and soyamilk like that of ferric chloride is well utilized in the rat. Once the iron is absorbed into the body it is not excreted in appreciable quantities and is hence available for hæmoglobin regeneration.

### SUMMARY

1. The hæmatopoietic property of autoclaved soyabean and soyamilk in young rats has been studied.

2. It is found that the ionizable iron in soyabean flour closely corresponds with the available iron. The soyabean milk is well utilized for the hæmoglobin formation.

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STUDIES ON ENZYME 'LIPASE': COMPARATIVE STUDY OF LIPASES  
OBTAINED FROM MOLDS GROWN ON OIL SEEDS

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In 1897, Camus (1) found that lipase is present in *Aspergillus Niger* and *penicillium glaucum*. In 1910, Dox (2) studied the enzyme activities of molds in mycelial stage. In 1921, Hahn (3) explored the possibility of fat synthesis by fungus and yeast enzymes. In 1923, Oshima and Church (4) studied the enzyme production by yellow green molds (*Aspergillus Flavus oryzae*). In 1926, Waksman and Robertson (5) found that the broken cells in the micro-organisms themselves may contain the enzymes. In 1935, David Kirsh (6) studied the different factors influencing the activity of fungus lipase. In 1944, Archibald Rayner (7) found that certain molds decompose the edible oils into fatty acids and finally into  $\text{CO}_2$  and  $\text{CH}_4$ . In 1947, Herissey and Fleury (8) have effected the synthesis of beta methyl D. glucoside by ferment powder prepared from *Aspergillus Niger*.

A detailed investigation has been carried out on the molds grown on oil seeds, with reference to their lipase production and different factors which control the growth of lipase producing molds as well as the enzyme activity of the lipase obtained from these molds.



## EXPERIMENTAL

First of all the experiments are carried out to grow the molds on oil seeds as well as to obtain them in a pure form. The following oil seeds are taken for the experiment:

1. Castor seed (*Ricinus Communis*); 2. Groundnut seed (*Arachis hypogea*); 3. Sesame seed (*Seasamum Indicum*); 4. Safflower seed (*Carthamus*); 5. Mustard seed (*Brassica nigra*); 6. Oil seed from Mysore (*Guizotia abyssinica*) and 7. Cotton seed (*Gossypium herbaceum*)—the oil seeds which are commonly used in different parts of India.

*Cultivation of molds on oil seeds:* The oil seeds are taken in different dishes and kept in a big desiccator containing  $H_2O$  at the bottom just sufficient to give a moisture content of less than 15 per cent. and an arrangement to regulate the flow of air.

Three varieties of seeds are kept—seeds without removing the outer skin, seeds after removing the outer skin and steamed seeds. On the 5th day, the molds begin to grow in the first and the second case. On the tenth day, molds begin to grow in the third case also.

It is found that the molds grow very rapidly in seeds without skin since they can attack the fat in the seeds with greater ease and the molds' growth is less in steamed seeds in the beginning since they are sterilized. Even though the molds grow on all types of seeds after some period, it is better to grow the molds on seeds with skin on since it will be more economical. So the different oil seeds with the skin on are treated as above and the molds are allowed to grow on them.

*Purification of molds:* The molds grown on oil seeds are cultured in artificial Czapek's medium in petri dishes and from this pure cultures are prepared.

Staining method used by Bayliss, Glick and Siem (9) as well as the biological method used by Grabill and Reed (10) was employed to detect the lipolytic molds from the pure cultures obtained. In general, black, green, yellow, blue and white molds grown on oil seeds are found to be lipolytic. The following pure cultures of molds which are lipolytic are identified and in this connection we take the opportunity to thank Dr. L. N. Rao, Professor of Botany, University of Mysore, who has taken great pains to identify these lipolytic molds: (1) *Aspergillus* Sp. (2) *Penicillium* Sp. (3) *Curularia* Sp. (4) *Rhizopus* Sp. (5) *Fusarium* Sp. (6) *Oospora* Sp. (7) *Hyphomycetes* Sp.

# STUDIES ON ENZYME 'LIPASE'

Factors which control the growth of molds:

The different factors which control the growth of lipolytic molds are studied.

*Effect of nature of the medium on the growth of molds:* It is necessary to find out the optimum conditions to grow the lipolytic molds on a large scale. The first factor is a suitable medium to grow these molds. Hence the following mediums are tried: (1) Czapek's liquid medium; (2) Raulin's liquid medium; (3) Pasteur's medium and (4) groundnut cake medium.

The first three media are prepared according to standard methods and sterilised. The fourth medium consists of 10 g. of groundnut cake in 250 cc. flask, moistened sufficiently with water and sterilised. The pure cultures of black, green and yellow lipolytic molds are inoculated in these media and incubated at 37°C. After two days, the molds begin to grow in them. On the third day, the flasks are removed and a general study is made on these molds.

In the Pasteur medium, the growth of the molds is very poor. In Czapek's medium, the black molds grow well whereas in Raulin's medium, the green molds grow well. In cake medium, all the molds grow well.

The black and green molds grown in Czapek, Raulin and cake medium are removed after three days, washed with water and then treated with petroleum ether to kill the cells. They are dried at room temperature in order to remove the solvent as far as possible since the prolonged contact of the solvent may destroy the activity of the lipase, powdered well and sieved through a 60 mesh sieve and preserved in a bottle after the addition of toluene to avoid bacterial growth and used for the experiment.

Each flask consists of 1 cc. of groundnut oil; 0.1 g. of lipolytic powder and 5 cc. of water incubated for 24 hours at 37°C. A blank always accompanied the sample. After the period of incubation, the contents of the flask is removed, 25 cc. of neutral alcohol are added and titrated against N/10 sodium hydroxide. The difference between the sample and the blank will give the activity of the lipase in terms of cc. of N/10 sodium hydroxide. The results are given in Table I.

TABLE I

Set No.	Name of the mold.	Difference in ccs. of N/10 sodium hydroxide between the sample and the blank for		
		Czapek's medium.	Raulin's medium.	Cake medium.
1	Black mold	2.8	1.2	2.5
2	Green mold	0.8	1.2	1.8

From the above table, it can be seen that lipases extracted from black and green molds grown in cake medium show a better activity. As it is observed that the molds grow well in the cake medium and the activity of the lipase extracted from the molds also show appreciable activity, the cake medium is used for further experiments.

*Selection of cake medium for mold growth:* The next point considered is the type of the cake to be used. An experiment is carried out to study the growth of molds by using different varieties of cakes for the medium. The ground nut cake is prepared from the Ghani method, expeller method as well as by extracting oil by cold press method. The three cakes are used for the experiment.

Each set of experiments consists of 10 g. of groundnut cake in 250 cc. flask, moistened sufficiently with water and sterilised. The black and the green molds are inoculated in these flasks and the flasks are incubated at 37°C. After two days, the molds begin to grow on them. On the third day, the flasks are removed and a general survey is made. The molds are removed, lipase is extracted and the activity of the lipase studied by standard method as in the previous case. The results are given in Table II.

TABLE II

Set No.	Name of the mold.	Difference in cc. of N/10 sodium hydroxide between the sample and the blank		
		Cold pressed cake	Ghani cake	Expeller cake medium
1	Black	3.1	2.9	2.5
2	Green	2.4	1.9	1.8

It is found that the molds grow well in cold pressed and ghani cake medium since the oil content in them is more than the expeller cake and further the activity of the lipase obtained from the molds in these two cases is also more. As ghani method is becoming popular in India, ghani cake is chosen as a medium for further experiments.

The next point to be considered is the selection of a good seed cake for the medium. So the cakes of all the different oil seeds are taken and the yellow mold is inoculated in different media containing different oil cakes.

Each set of the experiments consists of 10 g. of different oil seed cakes in 250 cc. flask moistened sufficiently with water and sterilised. The yellow mold is inoculated in these flasks and they are incubated at 37°C. for two days and on the third day, the flasks are taken out and a critical survey is made. It is found that a very good growth is observed in case of groundnut cake. The lipase is extracted in each case and the activity determined by standard method. The results are given in Table III.

TABLE III

Set No.	Name of the cake	Difference between sample and blank in terms of cc. of N/10 sodium hydroxide for the lipase extracted from mold.
1	Groundnut	2.0
2	Caster	0.9
3	Sesame	0.5
4	Mustard seed	0.5
5	Saffor	0.4
6	Cotton seed	1.7

## STUDIES ON ENZYME 'LIPASE'

From the above table, it can be seen that the groundnut cake medium is a suitable one to give an appreciably active lipolytic mold. Hence, the ground nut cake obtained by ghani method is used as a medium for growing molds.

*Effect of hion conc. of the medium on the growth of molds:* After finding out the suitable medium, the next factor to be considered is the Hion Con. of the medium since it plays a great role. So an experiment is carried out to find out the optimum pH for the medium to get a good growth of lipolytic molds. In different flasks, 10 g. of ghani cake of groundnut seed is taken, moistened sufficiently with water and adjusted to different pH varying from 4.4 to 8.0 pH by adding varying amounts to dil. HCl and then sterilised. Black and green molds are inoculated in them and kept in the incubator for two days at 37°C. On the third day, the flasks are removed and the growth of the mold is studied. The lipase is extracted in each case and its activity studied as usual. The results are given in Table IV.

TABLE IV

Set No.	Name of the mold.	Difference in ccs. of N/10 sodium hydroxide between the sample and the blank in case of medium of		
		5.2 pH.	5.8 pH.	6.0 pH
1	Black	0.8	2.9	0.5
2	Green	0.4	1.9	0.4

It is observed that the growth is maximum at 5.8 pH and also the lipase from mold grown in the medium of 5.8 pH shows maximum activity. Hence, the optimum pH of the medium for good active growth of lipolytic molds is 5.8 pH.

*Effect of concentration of the medium on the growth of lipolytic molds:* The next factor to be studied is the concentration of the medium. The experiment is carried out by taking different quantities of cakes in conical flasks moistened with sufficient water. The flasks are sterilised and then inoculated with the black and green molds incubated at 37°C for two days. On the third day, the flasks are removed and the molds examined. The results are given in Table V.

TABLE V

Set No.	Name of the mold.	Study of growths in the flask containing			
		10 g.	25 g.	50 g.	100 g.
1	Black	Growth	Good	Appreciably good	Very good growth
2	Green	"	"	"	"

It can be seen from the above table that more is the quantity of the cake used, more is the growth of the molds.

*Effect of change of moisture content:* The next important factor to be considered is the moisture content which is essential for the growth of molds. The experiment is carried out by taking 10 g. of cake in different flasks containing different

amount of water. The flasks are sterilised and then inoculated with the molds and incubated at  $37^{\circ}\text{C}$  for 2 days. On the third day, they are removed and the molds are examined. The lipases from the molds are prepared by usual method and their activity on groundnut oil is studied as usual. The results are given in Table VI.

TABLE VI

Set No.	Name of the Mold.	Difference in CCs. of N/10 sodium hydroxide between the sample and the blank for the sample containing the moisture content.		
		10 per cent.	15 per cent	20 per cent.
1	Black	0.8	2.9	0.9
2	Green	0.2	1.9	1.1

It is observed that the water content must be 15 per cent for good growth of the molds. From Table VI it is observed that the activity of the lipase is more in case of the lipase obtained from the mold grown in a cake medium containing 15 per cent water content. Hence the optimum moisture content for good growth of molds in the cake medium is 15 per cent.

*Effect of ageing on the growth of molds.* The next interesting factor is the effect of time on the growth of molds as well as the activity of the lipase extracted from them. An experiment is carried out to study the effect of ageing on the growth of molds.

Each experiment consists of 10 g. of groundnut cake in 250 cc. flask moistened with water and sterilised. The molds are inoculated in the flasks and the flasks are kept in the incubator at  $37^{\circ}\text{C}$  for different periods. At different time intervals, the flasks are removed and the mold growth is examined. The lipase is prepared from the molds and activity determined. The results are given in Table VII.

TABLE VII

Set No.	Name of the mold.	Difference in CCs. of N/10 NaOH between sample and blank on					
		1st	2nd	3rd	4th	5th	6th day of experiment.
1	Black	1.4	3.2	2.9	2.0	0.9	0.8
2	Green	0.5	2.1	1.9	0.8	0.3	0.3

It is found that the growth of mold increases with ageing. But from Table VII, it can be clearly observed that the activity of the lipase obtained from the mold increases only upto the second day and then begins to decrease gradually from the third day. Hence, even though the growth of the mold goes on increasing with time, the maximum activity of the lipase is obtained from the mold only on the second day. So it is better to grow the molds for two days in the cake medium and then extract the lipase from it.

Each set of the experiment consists of 10 g. of cake moistened sufficiently with water in a flask and sterilised. The flask is inoculated with the mold and air is passed at different intervals of time. The flasks are incubated at 37°C. for two days and then they are removed and the mold growth is examined. The lipases are prepared from these molds by usual method and their activity on the ground nut oil is studied and the results are given in Table VIII.

TABLE VIII

Set No.	Name of the mold.	Difference in CC. of N/10 sodium hydroxide between the sample and the blank when the time of aeration is		
		0 minutes ;	5 minutes ;	10 minutes
1	Black	3.2	7.8	0.4
2	Green	2.1	1.5	0.7

It is found that the aeration increases the growth of molds to appreciable extent. But from Table VIII, it is observed that lipolytic activity decreases with aeration and after ten minutes aeration, it may almost tend to zero. Hence, even though the aeration increases the growth of lipolytic molds, it retards the activity of the lipase extracted from the mold. So it is advisable to avoid aeration while growing lipolytic molds.

*Effect of temperature on the growth of molds:* An experiment is carried out to find the optimum temperature for the best growth of lipolytic molds in oil seed cake medium.

Each set of the experiments consists of 10 g. of cake in a flask moistened sufficiently with water and sterilised. The flasks are inoculated with the molds and incubated at different temperatures. After two days, the flasks are removed and examined for the growth of molds.

The lipases from the molds are extracted using standard methods and their activity on ground nut studied as usual. The results are given in Table IX.

TABLE IX

Set No.	Name of the mold.	Difference in CCs. of N/10 Sodium hydroxide between the sample and the blank for sample incubated at		
		32°C	35°C	37°C
1	Black	2.6	3.3	3.2
2	Green	1.5	2.4	2.1

It is found that the growth of the molds is appreciably good at the temperature 35 to 37°C. The maximum activity of the lipase is also obtained at 35 to 37°C. Hence, the optimum temperature for the growth of lipolytic mold is 35°C.

*Effect of different salts on the growth of molds:* The last factor to be considered is the effect of different salts on the growth of molds in a cake medium.

The salts taken for the experiment are sodium chloride, sodium nitrate, calcium chloride, ammonium sulphate, ferrous sulphate, citric acid, sodium phosphate, dil. HCl, acetic acid, alanine, triolein, ascorbic acid, glycerol. Decinormal solutions of the above substances are prepared and used for the experiment.

Each set of the experiments consists of 10 g. of cake in a flask moistened sufficiently with water, 1 cc. of salt solution and sterilised. The flasks are inoculated with the molds and incubated at 37°C for two days. Then they are removed and the mold growth is examined. The lipases from these molds are extracted using the standard method and their activities determined. The results are given in Table X.

TABLE X

Set No.	Name of the mold lipase.	Difference in CC. of N/10 Sodium hydroxide between the sample and the blank in case of				
		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	HCl	CH <sub>3</sub> COOH	Triolein	Glycerol.
1.	Black	0.9	0.2	0.0	1.0	0.8
2.	Green	0.6	0.4	0.1	1.3	1.3

It is found that generally organic salts accelerate the growth of molds. Out of inorganic salts, ammonium sulphate is the best accelerator and dil. HCl. and acetic acid are good. Out of the organic salts, glycerol and triolein are the best accelerators. Hence, it can be seen that even though organic compounds in general are good accelerators, carbon compounds like triolein and glycerol promote the growth of lipolytic molds to a very great extent. The lipases obtained from molds grown on the cake medium containing ammonium sulphate, triolein and glycerol are more active. So it is better to use organic compounds containing carbon as accelerators for promoting good growth of active lipolytic molds.

So from the above paragraphs it can be understood that black molds from castor and green mold from ground nut can be grown on ghani cake of ground nut with optimum pH-5.8 and moisture content 15% at 35 to 37°C. and the lipases can be extracted from them after two days.

*Different factors which control the activity of mold lipases :* It is observed that black mold grown on castor seed and green mold grown on ground nut show greater lipolytic activity and the optimum conditions for their maximum growth are studied.

These two molds are washed well with sterilised water and then treated with petroleum ether to kill the cells. Then they are completely dried at room temperature to remove the solvent adhering to it. They are powdered well, sieved through a 60 mesh sieve and preserved in bottles.

*Effect of the nature of the buffer, substrate and lipase on the activity of mold lipases:* The hydrolysis of different oils by the mold lipases using different buffers are carried out.

Each set of the experiments consists of 1 cc. of oil, 5cc. of water ; 0.1 g. mold lipase ; 2 cc. of buffer mixtures of varying pH value and incubated for the 24 hours at 37°C. Always a blank accompanied the sample. After the period of incubation the content is taken out and titrated against N/10 NaOH after the additions of 25 cc. of neutral alcohol and warming for sometime. Necessary precautions are taken to take readings under sterile conditions. The difference between the sample and the blank in terms of cc.s of N/10 sodium hydroxide will give the activity of the lipase. The results are given in Table XI to XIV.

TABLE XI

Lipase: Lipase extracted from black mold. Buffer: Disodium phosphate citric acid.  
Difference in ccs. of N/10 NaOH between the sample and the blank for

Set No.	Oil in ccs.	pH. value.	Castor oil.	G. Nut oil.	Sesame oil.	Safflower.	Mysore.	Mustard.	Cotton seed oil.
1	1.0	3.1	7.5	8.2	2.2	1.8	1.2	1.1	0.9
2	1.0	3.2	8.3	10.6	2.9	2.1	2.8	2.0	1.2
3	1.0	4.8	9.2	13.2	3.5	3.4	3.9	3.7	2.3
4	1.0	5.2	10.8	15.1	6.2	5.9	5.2	9.8	7.2
5	1.0	6.2	19.5	19.8	13.5	8.2	11.3	7.4	5.9
6	1.0	7.2	22.5	9.5	8.2	12.4	6.4	6.1	4.3
7	1.0	7.4	17.3	5.4	7.6	6.9	5.2	5.2	3.2
8	1.0	7.6	5.8	3.2	5.2	3.8	3.8	4.3	2.7

TABLE XII

Buffer: Disodium phosphate—citric acid.

Oil: Groundnut oil.

Difference in ccs. of N/10 NaOH between the sample and the blank for

Set No.	Oil in ccs.	pH. value.	Black mold.	Green mold lipase
1	1.0	3.1	8.2	5.6
2	1.0	3.6	10.6	8.9
3	1.0	4.8	13.2	10.9
4	1.0	5.2	15.1	12.2
5	1.0	6.2	19.8	18.8
6	1.0	7.2	9.5	1.7
7	1.0	7.4	5.4	2.1
8	1.0	7.6	3.2	1.2



TABLE XIII

BUFFER: Sodium phosphate—Potassium phosphate.			OIL: Groundnut oil. Difference in ccs. of N/10 NaOH between the sample and the blank for	
Set No.	Oil in ccs.	pH. value.	Black mold.	Green mold lipase.
1	1.0	5.28	9.6	10.6
2	1.0	5.90	10.8	12.3
3	1.0	6.46	13.4	15.6
4	1.0	6.97	12.2	13.8
5	1.0	7.16	10.1	11.1
6	1.0	7.38	7.3	9.6
7	1.0	7.73	5.2	7.6
8	1.0	8.04	3.1	5.4

TABLE XIV

BUFFER: Sodium hydroxide—Potassium phosphate.			OIL: Groundnut oil. Difference in ccs. of N/10 NaOH between the sample and the blank for	
Set No.	Oil in ccs.	pH. value.	Black mold.	Green mold lipase.
1	1.0	6.0	5.9	10.8
2	1.0	6.2	9.8	13.9
3	1.0	6.4	11.2	11.6
4	1.0	6.6	9.7	10.1
5	1.0	6.8	8.3	9.7
6	1.0	7.0	7.6	7.2
7	1.0	7.2	6.1	5.8
8	1.0	7.4	5.8	4.3

It can be seen that the optimum pH changes to a certain extent with the nature of the substrate as well as the nature of the lipase and nature of the buffer. Of all the lipases, lipase obtained from green mold grown on ground nut is the best since it shows the maximum activity in its own oil as well as appreciably good activity in other oils. The activity of the mold lipase is maximum in its own oil and groundnut oil is hydrolyzed appreciably by all mold lipases.

Since the optimum pH of the mold lipase is almost the same as that of Pancreatic Lipase, it is expected that this may behave like pancreatic lipase. Hence, it will be interesting to study these mold lipases in detail as they will be very useful in medicinal world to avoid the disease caused due to indigestibility of fat.

Hence it is observed that green mold from groundnut and black mold from castor are very active, groundnut oil is the best substrate and disodium phosphate-citric acid buffer is the best buffer.

*Effect of buffer concentration of the hydrolysis of groundnut oil by different mold lipases:* Each set of the experiments consists of 1 cc. of the groundnut oil, 5 cc. of water, 0.1 g. mold lipase and varying quantities of phosphate buffer of pH 6.2 incubated for 24 hours at 37°C. Always the blank accompanied each sample. After the period of incubation, the content is titrated against N/10 NaOH after adding 25 cc. of neutral alcohol and warming for sometime. Necessary precautions are taken to take readings under sterile conditions. The difference in terms of ccs. of N/10 NaOH between the sample and the blank will give the activity of the lipase. The results are given in Table XII.

TABLE XV

Set No.	Oil in ccs.	Ccs. of buffer added.	Difference in ccs. of N/10 NaOH between the sample and blank for	
			Castor.	Groundnut lipase.
1	1.0	1.0	14.2	17.1
2	1.0	2.0	19.8	18.8
3	1.0	3.0	15.2	16.8
4	1.0	4.0	12.8	14.3
5	1.0	5.0	10.1	10.2
6	1.0	6.0	9.2	8.0
7	1.0	7.0	7.3	8.0
8	1.0	8.0	6.1	7.1

From the above table, it can be seen that the maximum activity is obtained when the buffer concentration is 2 cc. Hence, the optimum buffer concentration for enzymic hydrolysis of the oil is 2 cc.

*Effect of substrate concentration on the hydrolysis of groundnut oil by different mold lipases:* Each set of the experiments consists of varying quantities of groundnut oil, 2cc. of phosphate buffer of 6.2 pH, 5 cc. of water and 0.1 g. of lipase incubated for 24 hours at 37°C. After the period of incubation, the contents is titrated against N/10 NaOH after the addition of 25 cc. of neutral alcohol and warming for sometime. Always each set is accompanied by a blank. Necessary precautions are taken to take reading under sterile conditions. The difference in ccs. of N/10 sodium hydroxide between the sample and the blank will give the activity of the lipase. The results are given in Table XVI.

From table XVI, it can be seen that the maximum activity is obtained when the substrate concentration is 2 to 3 cc. and hence the optimum substrate concentration is 2.3 cc. depending upon the nature of the lipase.

TABLE XVI

Set No.	Oil in ccs.	Difference in ccs. of N/10 NaOH between the sample and the blank for	
		Castor.	Groundnut lipase.
1	1.0	19.8	18.8
2	2.0	20.6	21.9
3	3.0	25.3	17.4
4	4.0	19.9	15.2
5	5.0	17.6	12.6
6	6.0	15.2	9.9

*Effect of enzyme concentration on the hydrolysis of groundnut oil by different mold lipases:* Each set of the experiments consists of 1 cc. of groundnut oil, 2 cc. of buffer, 5 cc. of water and varying quantities of the enzyme lipase and incubated at 37°C for 24 hours. After the period of incubation, the content is titrated against N/10 NaOH after the addition of 25 cc. of neutral alcohol and warming for sometime. Always a blank accompanied the sample. Necessary precautions are taken to take readings under sterile conditions. The difference between the sample and the blank in terms of ccs. of N/10 NaOH will give the activity of the lipase. The results are given in table XVII.

TABLE XVII

From the above Table, it can be seen that the percentage hydrolysis of the oil goes on increasing with the concentration of the enzyme. Greater the concentration of the enzyme used, greater is the activity of the mold lipase.

*Effect of temperature on the hydrolysis of groundnut oil by different mold lipases:* Each set of the experiments consists of 1 cc. of groundnut oil ; 5 cc. of water ; 2 cc. of Buffer and 0.1 g. of lipase kept at various temperatures in the incubator for 2 hours. After the period of incubation, the contents is titrated against N/10 sodium hydroxide after adding 25 cc. of neutral alcohol and warming for some time. Always a blank accompanied the sample. The difference between the sample and the blank in terms of cc. of N/10 sodium hydroxide will give the activity of the lipase. The results are given in Table XVIII.

TABLE XVIII

Set No.	Temperature in °C.	Difference in ccs. of N/10 NaOH between the sample and the blank for Groundnut mold lipase.
1	28.0	5.2
2	30.0	5.9
3	35.0	9.6
4	37.0	12.4
5	40.0	10.4
6	75.0	2.2
7	100.0	0.0
		3.8
		3.8
		10.7
		11.8
		9.6
		1.3
		0.0

From the above table, it can be seen that percentage hydrolysis is maximum at 37°C. So the optimum temperature is 37°C. It will be interesting to note that the optimum temperature for growth of lipolytic molds as well as for the hydrolysis of mold lipase is the same.

*Effect of salts on the hydrolysis of groundnut oil by mold lipases:* Each set of the experiments consists of 1 cc. of groundnut oil ; 2 cc. of phosphate buffer ; 5 cc. of water ; 0.1 g. of lipase and 0.1 g. of salt (or 1 cc. of N/10 salt solution in case of hydrochloric acid and acetic acid) incubated at 37°C for 24 hours. A blank always accompanied the sample. After the period of incubation, the contents is titrated against N/10 NaOH after adding 25 cc. of neutral alcohol and warming for sometime. The difference will show whether a blank will give the activity of the lipase. The difference depending upon whether the difference is a positive one (showing the accelerating effect) or a negative one, showing the retardating effect. The results are given in Table XIX.

TABLE XIX

Set No.	Name of the salt.	Difference in ccs. of N/10 NaOH between the sample and the blank for	
		Castor.	Groundnut mold lipase.
1	Albumin	+ 0.2	+ 0.8
2	Sodium Taurocholate	+ 3.2	+ 4.9
3	Gum Arabic	+ 1.2	+ 0.8
4	Ascorbic Acid	+ 1.5	+ 0.5
5	Citric Acid	+ 2.8	+ 0.3
6	Acetic Acid	+ 2.3	+ 1.3
7	Strychnine Chloride	- 2.5	- 1.2
8	Strychnine sulphate	+ 0.3	- 0.9
9	Glycine	- 1.0	+ 1.4
10	Sodium acetate	+ 2.2	+ 2.4
11	NaCl	+ 1.8	+ 1.2
12	NaNO <sub>3</sub>	- 2.1	- 1.3
13	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	+ 1.2	+ 1.7
14	Mn SO <sub>4</sub>	+ 2.3	+ 2.5
15	Na <sub>2</sub> PHO <sub>4</sub>	+ 1.4	+ 1.2
16	KH <sub>2</sub> PO <sub>4</sub>	+ 2.3	+ 4.8
17	CaCl <sub>2</sub>	- 1.2	+ 0.7
18	HCl	+ 2.6	+ 1.6

From the above table, it can be seen that a definite conclusion cannot be drawn as regards the effect of the nature of the salt on the activity of the lipase since the same salts accelerates in one case and sometimes retards in another case. From the organic salts, sodium taurocholate, gum arabic and sodium acetate are the best accelerators and ammonium sulphate, KH<sub>2</sub> PO<sub>4</sub>, Mn SO<sub>4</sub> and HCl are the best accelerators out of the inorganic salts. Further the salts used for preparing buffer solutions do not retard the lipase activity.

#### COMPARISON BETWEEN DIFFERENT PREPARATIONS OF LIPASE AS REGARDS THEIR ACTIVITY

Since the activity of the lipase prepared by Longnecker's method goes on decreasing on ageing, different preparations of the lipase are made and their activity on ageing studied in order to get a sample which keeps its activity appreciably constant for a fairly long time.

Each set of the experiments consists of 1 cc. of groundnut oil, 5 cc. of  $H_2O$ , 2 cc. of phosphate buffer and 0.1 g. of lipase of each variety at different intervals of time kept in the incubator at  $37^\circ C$  for two hours and then titrated against N/10 NaOH after adding 25 cc. of neutral alcohol and warming for sometime. The difference between the sample and the blank gives the activity of the lipase in terms of ccs. of N/10 NaOH. The results are given in Table XX.

TABLE XX

Set No.	Time of ageing.	BUFFER: Disodium Phosphate—Citric acid.		LIPASE: Castor Mold Lipase.	
		Difference in ccs. of N/10 NaOH between the sample and blank for		Petroleum ether and acetone mixture.	
		Petroleum ether dried.			Acetone dried sample.
1	0 minutes				
2	24 Hours	10.8			
3	2 days	12.4		9.6	8.5
4	4 "	13.9		11.8	9.2
5	8 "	15.2		12.3	11.2
6	12 "	11.6		14.3	11.8
7	15 "	10.8		10.6	10.5
8	23 "	9.9		9.3	9.8
9	45 "	9.1		9.0	9.2
10	55 "	8.8		7.8	8.6
11	65 "	8.5		6.9	8.1
12	75 "	8.2		6.3	7.9
13	85 "	7.9		6.1	7.5
14	95 "	7.2		5.8	7.0
15	105 "	6.8		5.5	6.3
16	115 "	6.2		4.9	6.0
17	125 "	5.2		4.0	6.0
		4.4		3.8	5.9

From the above table, it can be seen that in case of petroleum ether and petroleum ether and acetone mixture dried sample, the activity increases upto 2-4 days and then slowly decreases. In case of acetone dried sample, the activity is less but the change in activity is not so vigorous as in other cases. Hence acetone dried sample can be used since it is stable for a fairly long time.

So it can be observed that the mold lipases can be used in the industrial as well as medicinal world if a method is worked out to prepare it in large scale.

#### SUMMARY

The different factors which control the growth of lipolytic molds as well as the activity of the mold lipases are studied in detail.

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INFLUENCE OF DIETARY FATS ON CERTAIN CONSTITUENTS OF LIVER,  
BLOOD AND BODY OF ALBINO-RATS WITH SPECIAL  
REFERENCE TO FAT UTILIZATION

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Growth experiments on rats have been extensively carried out to show the relative nutritive values of various dietary fats. Deuel *et al* (1) found no difference in the growth of weanling rats fed on an adequate diet in which the fat component was butter, margarine, groundnut, maize, cotton-seed or soya-bean oils. Even when reproduction and growth-rates were studied over ten generations, Deuel *et al* (2) found that margarine could adequately replace butter. Henry *et al* (3) concluded from their rat-growth experiments that it is unlikely that butter-fat possesses nutritive properties superior to those of other fats. On the other hand, Boer *et al* (4) reported that the growth with summer-butter-fat was slightly but significantly greater than with groundnut, olive and rape-seed oils. The substance responsible for the superior growth was traced (5) to vaccenic acid. Later, however, Jansen (6) stated that consistent results were not obtained with purified vaccenic acid. The recent reports of Deuel *et al* (7), Nath *et al* (8) and Euler *et al* (9) would seem to establish that vaccenic acid has no growth-stimulating action in rats.

The digestibility of various types of fats and oils has been the subject of very extensive investigation (10-18). The general trend of evidence would show that most of the commonly used animal and vegetable fats including some hydrogenated fats (with the probable exception of those few having large proportions of stearic acid) are digested to essentially the same degree of completeness.

Very little work has been done on the comparative composition of rats fed with different fats. Deuel *et al* (19) determined the composition in terms of water, protein, lipid, carbohydrate, ash and calcium of bodies of rats receiving different fats. Loeb and Burr (20) reported the composition in terms of total lipid, non-lipid solids and water-content of rats raised on fat-free and fat-supplemented diets.

The present study has been designed to determine whether there are any differences (i) between the growth-rates of rats receiving refined groundnut oil, *versus* p.c.o.



(of M.P. 37°C) made out of it or cow-ghee as the main source of fat, (ii) between the degrees of utilization of dietary fats, and (iii) between certain constituents of liver, blood and body of rats raised on these fats.

### EXPERIMENTAL

Sixty-four litters (32 males plus 32 females) were weaned after 28 days when they weighed 34 to 43 g. They were placed for four weeks on a practically fat-free synthetic diet composed of corn-starch (65%), extracted casein (15%), sugar (10%), yeast (5%) and Osborne and Mendel (21) salt-mixture (5%). They were then divided into four groups of 16 each (8 males and 8 females)—Group A was allowed to continue for twelve weeks more with the same fat-free diet and the other three groups were put for twelve weeks on the experimental diet composed of corn-starch (20%), extracted casein (30%), sugar (10%), fat (30%), Yeast (5%) and salt-mixture (5%)—Group B receiving refined groundnut oil; Group C, vanaspati (of M.P. 37°C) prepared out of it and Group D, cow-ghee. The fat-free diet as well as the fat-supplemented diets were supplemented with vitamins A, D and E, each receiving daily 60 I.U. of vitamin A and 10 I.U. of vitamin D and 0.5 mg. of toco-pherol acetate in propylene glycol. In the case of the ghee group, the vitamin A that was already present in the ingested ghee was taken into account in supplementing the diet with vitamin A. The protein content of the fat-supplemented diets was increased from 15% to 30% as it has been well-established (22) that ingestion of high fat and low-protein diet results in the production of dietary fatty livers, irrespective of the nature of the fat used.

The rats of each group were given *ad libitum* feeding. Weekly records of weights of rats during the experimental period were maintained. The results are given in Table I. For each group, the faeces were collected in bulk under alcohol for the entire experimental period at the end of which, the faecal fat was extracted and used for a separate study on the fatty acids composition of faecal fat, the results of which will be reported in subsequent communications.

When the rats reached the 12th week on the experimental diet, metabolism studies were carried out. For five days, the faeces were collected individually, pooled in groups of four from the same series and sex, dried and analysed for fat by Soxhlet extraction with ether so as to determine the digestibility of the fats. The results are shown in Table II.

At the end of the experimental period, the rats were anaesthetized and the blood was withdrawn from the abdominal aorta. The blood-samples were pooled in the same groups of four as mentioned before in test-tubes containing a few crystals of sodium citrate and immediately centrifuged. The total cholesterol and the total fatty acids in the plasma separated were determined by Bloor's method (23) and for the determination of the iodine number of plasma fatty acids, Yasuda's method

acids by Bloor's method (23) and Yasuda's method (24) was followed for the determination of iodine number of liver fatty acids. For Vitamin A assay in the liver, Moore's method (25) of extraction of Vitamin A from the alkaline digests of liver was followed and the intensity of blue colour developed with antimony-trichloride reagent was measured in Pulfrich Photometer using filter S.61. The Vitamin A content was expressed as  $B_{500}^{1\%}$  as suggested by Jensen and With (26). The results on liver and blood analyses are shown in Table III.

The gastro-intestinal tracts were discarded and the combined bodies of the same groups of four rats as mentioned before were finely chopped with a meat-chopper, minced into a pulpy mass in a meat-mincer and homogeneously mixed. A suitable aliquot of the minced mass was dried and analysed for fat by Soxhlet extraction with ether. Body-fat was extracted from the rest using alcohol and ether. The entire defatted body was dried and finely ground in a mechanical grinder. One-fourth of the powder was dry-ashed by the method adopted by Sherman and MacLeod (27) and on the ash-solution, calcium was determined by the procedure of Shohl and Pedley (28). Iodine value of the extracted body-fat was determined by Rosenmund and Kuhnheim's method as described by Vere-zones (29). Per cent of body-fat and body-calcium were calculated on the basis of wet weight of the body minus the gastro-intestinal tract and liver which were removed. The results of body analyses are given in Table II.

The combined body-fat from each series was analysed for detailed fatty acids composition, the results of which will be reported in subsequent communications.

TABLE I  
*Growth-rates of rats on synthetic diet without or with fat supplements  
over a period of 12 weeks*

(Average values of 8 rats)					
Group	Fat used.	Rat No. and sex.	Weight after 4 weeks on fat-free diet g.	Weight after 12 weeks on experimental diet g.	Average weekly increase in weight g.
A	No. fat	1 to 8 M	82.3	188.1	8.8 ± 0.51*
		9 to 16 F	76.2	144.5	5.7 ± 0.43
B	Refined groundnut oil	1 to 8 M	83.8	187.4	8.6 ± 0.38
		9 to 16 F	75.4	150.1	6.2 ± 0.54
C	Vanaspati (M.P. 37°C)	1 to 8 M	83.2	189.0	8.0 ± 0.50
		9 to 16 F	75.9	148.0	6.0 ± 0.49
D	Cow-ghee	1 to 8 M	82.0	192.0	8.1 ± 0.44
		9 to 16 F	75.1	181.3	6.4 ± 0.45

\*Standard error of the mean.

TABLE II

*Digestibility of ingested fats in adult rats ; body-fat and body-calcium of rats raised on synthetic diet without or with fat supplements*

(Average values of four rats)

Group.	Rat No. and Sex.	Food intake in the metabolism period of 5 days g.	Fat intake in 5 days g.	Dry wt. of faeces in 5 days g.	Faecal fat in 5 days g.	Digestibility per cent.	Body fat per cent.	Iod. val. of body fat.	Body calcium per cent.
A	1 to 4 M	78.5	0.30	6.78	0.21	—	11.02	58.1	0.70
	5 to 8 M	77.8	0.29	6.60	0.18	—	11.54	59.8	0.73
	9 to 12 F	67.2	0.25	6.18	0.22	—	11.28	58.7	0.81
	13 to 16 F	68.9	0.26	6.21	0.14	—	11.91	59.6	0.82
B	1 to 4 M	72.2	21.66	6.92	0.76	96.47	13.81	81.2	0.88
	5 to 8 M	73.3	21.99	6.71	0.81	96.32	14.90	84.9	0.90
	9 to 12 F	61.8	18.54	6.20	0.79	95.74	14.12	80.3	1.02
	13 to 16 F	62.2	18.66	6.33	0.80	95.72	14.33	80.9	0.99
C	1 to 4 M	71.9	21.57	6.81	1.04	95.19	15.24	72.4	0.89
	5 to 8 M	74.8	22.44	7.20	1.19	94.69	13.95	72.0	0.92
	9 to 12 F	65.0	10.50	6.40	1.18	93.97	15.01	71.7	1.01
	13 to 16 F	61.2	18.36	6.61	1.08	94.10	14.02	72.5	0.97

TABLE III

*Liver and blood analyses of rats raised on synthetic diet without or with fat-supplements*

(Average values of four rats.)

Group.	Rat No. and Sex.	Wt. of Fresh liver g.	Per cent fresh liver.		Iod. val. of liver fatty acids.	Liver vitamin A & B <sub>1</sub> cm	Per 100 cc. of blood plasma.		Iod. val. of plasma fatty acids.
			Total Cholesterol.	Total fatty acids.			Total Cholesterol mg.	Total fatty acids mg.	
A	1 to 4 M	7.12	0.190	3.01	120	0.169	68	253	87
	5 to 8 M	6.97	0.206	3.20	116	0.181	80	282	95
	9 to 12 F	6.23	0.184	3.15	119	0.158	72	231	91
	13 to 16 F	6.30	0.216	3.24	114	0.180	86	270	98
B	1 to 4 M	6.99	0.201	3.25	118	0.187	88	297	136
	5 to 8 M	7.23	0.182	3.49	122	0.159	102	334	140
	9 to 12 F	6.42	0.188	3.37	118	0.192	98	301	146
	13 to 16 F	6.31	0.200	3.31	115	0.160	109	292	132
C	1 to 4 M	7.24	0.219	3.10	117	0.168	108	291	130
	5 to 8 M	7.10	0.190	3.28	119	0.179	87	330	103
	9 to 12 F	6.38	0.214	3.20	119	0.172	112	286	121
	13 to 16 F	6.21	0.192	3.31	115	0.181	92	296	117
D	1 to 4 M	7.30	0.204	3.09	120	0.157	90	311	105
	5 to 8 M	7.16	0.210	3.25	117	0.190	101	287	133
	9 to 12 F	6.19	0.221	2.68	118	0.174	111	332	128
	13 to 16 F	6.37	0.184	3.37	120	0.168	102	300	119

TABLE IV

*Relationship between the Iodine value of dietary fat and that of body, liver and plasma—lipids of rats*

Name of dietary fat.	Iod. val. of dietary fat.	Average Iod. value of Body-fat.	Average Iod. value of plasma fatty acids.	Average Iod. value of liver fatty acids.
No fat	—	59.0	92.7	117.2
Refined groundnut oil	93	81.8	138.5	118.2
Vanaspati (of M.P. 37°C)	66	72.1	117.7	117.5
Cow-ghee	41	62.0	119.7	118.7

## DISCUSSION

Table I shows that the average weekly increase in weight of rats receiving synthetic diet without or with the different fat-supplements is practically of the same order and the influence of refined groundnut oil, Vanaspati (37°C) and ghee on promotion of growth of young rats is practically the same. Deuel *et al* (30) have shown that on a really fat-free diet, growth of rats is poorer than on diets supplemented with fats. The diet used in the present work, however, was not rigidly fat-free. Although casein used was extracted thrice with alcohol, the diet in bulk was not absolutely fat-free.

Burr and Burr (31) and McAmis *et al* (32) simultaneously reported that normal growth is impaired and characteristic fat-deficiency symptoms develop in rats fed on diets adequate in all respects but rendered rigidly fat-free. Burr and Burr (33) showed further that linoleic acid and possibly other unsaturated acids can cure this fat-deficiency disease. In the present experiments, however, rats receiving practically fat-free diet did not show any fat-deficiency symptom. This can be explained by the fact that the diet used was not rigidly fat-free, it contained 0.38 per cent ether-extractives. Moreover, it has been reported (34) that corn-starch contains about 0.6 per cent of fatty material which is within the granule and is non-extractable. It has also been shown (35, 36) that yeast contains 2.5 to 4.0 per cent of lipids which are firmly bound and non-extractable by ether. Evans and Lepkovsky (37) have further observed that trace of fatty acids in corn-starch, about 0.5 per cent, is sufficient to cure the fat-deficiency disease of rats in a spectacular manner.

Practically no difference in the percentage of body-fat is noted between the three groups of rats receiving refined groundnut oil, Vanaspati (37°C) or ghee. The body-fat content of rats raised on fat-free diet, however, appears to be slightly lower than that of the fat-supplemented groups. There is no significant sex-difference in the deposition of fat in the body of rats raised on fat-free or fat-supplemented diets. Boycott and Demant (38) stated that female rats tend to have a slightly higher fat content. Deuel *et al* (19) also have reported that female rats have a slightly higher fat content than the males. On the other hand, Bachmann *et al* (39) found no appreciable difference in fat-contents of male and female rats. Loeb and Burr (20) also could not find a significant sex-difference in the storage of fat in normal rats.

The unsaturation of body-fat of rats when compared with that of the corresponding dietary fat, shows that the fat stored in the body is influenced to a great extent by dietary fat. Anderson and Mendel (40) reported a close parallelism between the iodine number of body-fat and that of food-fat when the latter furnished 60 per cent of the total energy-intake. The iodine value of the body-fat of rats raised on groundnut oil diet has been found to be slightly higher than that of butter-fat group or Vanaspati group. During the process of absorption and deposition, however, it appears that the ingested fat may undergo desaturation or saturation. These are indicated respectively (i) from the higher iodine value (62.0) of the body-fat as compared with the iodine value (41) of the ingested butter-fat and (ii) from the lower iodine value (81.8) of the body-fat as compared with the iodine value (93) of the ingested groundnut oil. On the fat-free diet, fat of comparatively lower iodine value is synthesized and deposited.

With regard to calcium deposited in the body of rats, the storage appears to be slightly lower in rats on fat-free diet than on the fat-supplemented diets. The calcium-content of the body of rats raised on groundnut oil, Vanaspati or ghee is practically identical. The percentage of calcium deposited in females appears to be slightly higher than that in male rats. The difference in calcium-contents of male and female rats is practically of the same order as that reported by Sherman and MacLeod (27), Sherman and Booher (41), Lanford *et al* (42) and Deuel *et al* (19). The latter authors consider this small sex-difference as significant.

The results on blood analysis show that there is practically no difference in the concentration of total cholesterol or total fatty acids in the blood-plasma of rats raised on groundnut oil, vanaspati or ghee diet. The unsaturation of plasma fatty acids of rats receiving groundnut oil has been found to be slightly higher than that for rats receiving vanaspati or ghee. The degree of unsaturation of the plasma-fatty acids and the values for total cholesterol or total fatty acids on fat-free diet are lower than the corresponding values on the fat-supplemented diets. A distinct fall in the degree of unsaturation of the serum-fatty acids as well as in the concentrations of total cholesterol and total fatty acids in the blood of experimental animals on an extremely low-fat diet has been consistently reported (33, 43, 44, 45). A tendency for the iodine number of the serum-lipoids to vary directly and for the total amount of serum-lipoids to vary inversely with the iodine number of the dietary fat has also been reported (44, 46, 47).

The values for total cholesterol and total fatty acids of the liver and iodine value of liver fatty acids have been found to be more or less the same in the fat-free group as well as in the fat-supplemented groups and fall within the normal range. The normal deposition of liver-fat has not been affected by the nature of the dietary fats studied.

The analyses of liver-vitamin A reveal no significant difference between the fat-free group and the fat-supplemented groups. Storage of vitamin A in the liver has not been influenced by the ingestion of any of the three fats studied. Thorbjarnarson and Drummond (48) found that rats receiving no other fat than 3.6 mg. cod liver oil daily stored normal amounts of vitamin A in livers. Sobel *et al* (49) compared vitamin A liver-storage in rats and observed that vitamin A is far better absorbed from aqueous media than from an oily solution.

The evidence regarding sex-difference in vitamin A storage is conflicting. Baumann *et al* (50) and Lemley *et al* (51) reported that there is no sex-difference, whereas Brenner *et al* (52) observed that under comparable experimental conditions females store more vitamin A in the liver than males. In the present study, no sex-difference in vitamin A storage is observed.

#### SUMMARY

(1) Four groups of rats were raised on synthetic diet without or with different fat-supplements viz., refined groundnut oil, vanaspati (of M.P. 37°C) made out of it and cow-ghee. No significant difference in the growth-rates of rats of the four groups was observed. No fat-deficiency symptom developed in rats of the fat-poor group.

(2) The digestibility of the three fats was found to be practically identical and was of the order of 95 per cent.

(3) Practically no difference in the percentage of body-fat is noted between the three fat-supplemented groups. The body-fat content of rats of fat-free group appears to be slightly lower than that of the fat-supplemented groups. The unsaturation of the body-fat of rats when compared with that of the corresponding dietary fat, shows that the fat stored in the body is influenced to a great extent by dietary fat.

(4) The storage of calcium appears to be slightly lower in rats on fat-free diet than on the fat-supplemented diets. The calcium-content of the body of rats receiving different fat-supplements is practically identical.

(6) The values for total cholesterol and total fatty acids of the liver and iodine value of liver fatty acids are practically of the same order in the fat-free group as well as in the fat-supplemented groups and fall within the normal range. The analyses of liver-vitamin A reveal no significant difference between the fat-free group and the fat-supplemented groups.

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DESTRUCTION OF VITAMIN A AND ATTENDANT CHANGES  
FOLLOWING THE ABSORPTION OF OXYGEN BY  
SHARK-LIVER OIL

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The development of peroxides and destruction of vitamin A in liver-oils exposed to atmospheric conditions have been reported by Rosenheim and Webster (1), Wokes and Willimott (2), Whipple (3), Lowen *et al* (4) and Rao (5). No study, however, has been made on the measurement of the amount of oxygen absorbed in liver-oils and the changes of their analytical characteristics along with the absorption of oxygen. In this paper, the amount of oxygen absorbed in shark liver oil exposed to oxygen has been measured at different stages of autoxidation and the effect of absorption of oxygen on the destruction of vitamin A and the changes of the important chemical characteristics has been studied.

EXPERIMENTAL

The volume of oxygen absorbed in shark liver oils was measured in the same apparatus as had been previously used by Bose *et al* (6) in their experiments on the absorption of oxygen by lemon grass oils. The apparatus used was a modification of that used by Bose (7) in his experiments on the absorption of oxygen by turpentine oil. The modified apparatus could be evacuated and refilled with oxygen.

Eight grams of oil were accurately weighed into the reaction-flask which was then closed and mercury-sealed. The flask as well as the graduated tube was

evacuated to a very low pressure from a Cenco-pump and then filled with dry oxygen. The flask was half-immersed in an oil thermostat previously adjusted at the selected temperature. Any excess pressure was released by momentarily opening the stop-cock. The absorption of oxygen was indicated by the gradual rise of the mercury-column. The heights of the mercury-column (after being adjusted for atmospheric pressure) and barometric readings were noted at suitable intervals. Temperature and barometric corrections were applied in order to get the exact volume of oxygen absorbed at N.T.P. and the weight (in g.) of oxygen absorbed was calculated, on the basis that one litre of oxygen at N.T.P. weighs  $(10 \times 0.0898)$  g.

A possible disadvantage of this method is that the rate of oxygen-absorption *may be influenced by pressure-changes*. To obviate this, the system was periodically re-adjusted by admitting more oxygen into the reaction-flask and occasionally, the flask was taken out of the oil-bath, evacuated after cooling the oil and re-filled with dry oxygen. Another objection may be raised for the oil being not stirred during exposure. A thin layer of oil exposed, however, can allow sufficient penetration of oxygen.

The oil used was rigorously dried on shaking with sufficient amount of anhydrous sodium sulphate and filtering the supernatant oil through a column of a fresh amount of anhydrous sodium sulphate under suction. While treating the oil with sodium sulphate, it was maintained at about  $18^{\circ}\text{C}$  for effective dehydration.

The first set of experiments were carried out at  $37^{\circ}\text{C} \pm 0.2$ , the second set at  $100^{\circ}\text{C} \pm 0.2$  and the other sets at  $37^{\circ}\text{C} \pm 0.2$  but under different conditions. In all these experiments, the top of the flask was covered with black paper so as to avoid any effect of light.

For the third set of experiments, the fatty-acids were isolated from the same sample of oil by the usual method of saponification and the subsequent acidification of the soaps and the required amount of the washed and dried fatty acids was added to it so as to raise the acid value to about 5—the figure usually observed (8) in commercial samples of oil. 1.0 per cent (by volume) distilled water, which is usual moisture content of commercial samples as reported before (8) was then intimately mixed with the oil of raised acidity.

The destruction of vitamin A and the changes of the chemical characteristics in the oil were studied at five different stages of autoxidation. In the first experiment of each set, the maximum amount of oxygen that the oil could absorb under the experimental conditions was noted and in other four experiments, the periods of exposure were so controlled as to obtain four samples of oil saturated with approximately one-fifth to four-fifths of the total amount of oxygen as absorbed in the first sample. Each sample was analysed for acid value, iodine value, saponification value, acetyl value, peroxide number and vitamin A content. Iodine value was determined by Rosenmund and Kuhnheim's method as described by Vere-Zones (11). Peroxide number was determined by Wheeler's method (12) and was expressed as ml. of I.O.N. thiosulphate per kilogram of oil. Standard methods (13) were followed for the determination of acid, saponification and acetyl values. The samples containing 1.0% moisture were analysed (14,8) for vitamin A in Hilger Vitameter A and the rest were analysed (15,8) by Carr-Price reaction in Pulfrich Photometer using filter S.61. Since only 8 g. of oil were available for all these analyses, 4 g. were used for acetyl value and 2 g. for acid value (using micro-burette in each case) and the remaining oil was sufficient for the other estimations. The results of the analyses are given in tables II to V. In each experiment of each set, the initial period (Induction period) during which there was no absorption of oxygen was also noted and the mean values are given in table I.

TABLE I

*Induction period of shark liver oil under different conditions.*

Sample of Oil.	Induction period (hours) at	
	37°C	100°C
Moisture-free oil (acid value, 0.71)	22.4	0.2
Oil (acid-value, 5.48) + moisture (1.0%)	16.0	—
Oil + dissolved copper (0.0021%)	4.6	—

TABLE II

*Oxygen-absorption at 37°C, destruction of vitamin A and changes of analytical characteristics.*

Period of exposure. hrs.	Ml. of oxygen ab- sorbed at N.T.P.	Wt. of oxygen ab- sorbed, g.	Acid value.	Iodine value.	Sap. value.	Acetyl value (apparent).	Peroxide No.	Vit. A, I.U. B 1cm.	Per cent in Vit. A
0	—	—	0.71	148.1	165.2	12.92	0	7.39	—
45	131.8	0.1892	0.74	136.1	..	13.08	26.5	7.64	23.0
68.5	264.5	0.3797	0.82	121.3	..	13.84	56.5	4.33	11.4
99.5	390.7	0.5610	0.96	99.8	109.6	14.97	94.3	3.22	5.4
133	529.1	0.7508	1.00	78.6	167.0	16.29	139.8	2.84	2.4
168	654.6	0.9300	1.21	52.2	168.3	17.65	162.2	—	—

TABLE III  
*Oxygen-absorption at 100°C, destruction of vitamin A and changes of analytical characteristics.*

Period of exposure. hrs.	Ml. of oxygen ab- sorbed at N.T.P.	Wt. of oxygen ab- sorbed, g.	Acid value.	Iodine value.	Sap. value.	Acetyl value (apparent).	Peroxide No.	Vit. A 1% B 1 cm.	Loss in Vit. A per cent.
0	—	—	0.71	148.1	166.2	12.92	0	7.39	—
8.5	138.2	0.1985	0.78	132.3	..	13.71	18.4	4.97	32.7
18	277.9	0.3990	0.97	114.5	..	15.14	22.5	3.05	58.7
27	420.2	0.6033	1.29	93.3	166.6	16.70	14.2	1.67	77.4
40.5	556.1	0.7986	1.76	68.7	167.9	18.33	11.8	—	—
53.5	697.7	1.0020	2.09	41.4	168.9	19.83	8.4	—	—

TABLE IV  
*Oxygen-absorption at 37°C on addition of fatty acids and moisture, destruction of vitamin A and changes of analytical characteristics.*

								Vit. A 1% E 1 cm.	
0	—	—	5.48	150.2	167.1	15.25	0	4.25	—
38.5	134.0	0.1924	5.67	136.9	..	15.61	29.8	3.11	26.8
63.5	264.1	0.3792	5.88	123.0	..	16.80	63.2	2.63	38.1
90	399.0	0.5729	6.29	96.8	167.5	18.39	111.9	1.70	60.0
122.5	531.8	0.7635	6.64	75.2	168.6	19.51	156.2	1.03	75.8
151	670.8	0.9632	7.20	48.6	169.0	20.62	186.8	—	—

TABLE V  
*Oxygen-absorption at 37°C in presence of traces of dissolved copper, destruction of vitamin A and changes of analytical characteristics.*

								Vit. A 1% B 1 cm.	
0	—	—	0.69	148.1	166.2	12.92	0	6.89	—
28.5	129.9	0.1865	0.65	140.3	..	12.92	22.3	5.15	25.2
52	260.1	0.3736	0.65	124.5	..	13.18	41.3	4.01	41.8
79	388.9	0.5584	0.74	101.8	166.4	14.27	62.8	2.62	62.0
107.5	525.2	0.7543	0.89	76.6	167.5	15.33	46.7	1.48	78.5
138	650.7	0.9344	1.04	53.7	167.9	16.54	30.2	—	—

## DISCUSSION

The total amount of oxygen absorbed in shark liver oil at 37°C was practically the same, irrespective of the conditions under which it was exposed. At 100°C, slightly more oxygen was absorbed. The induction period as well as the period of exposure during which the maximum amount of oxygen was absorbed, however, varied with the conditions of exposure. To state more clearly, 0.94 g. of oxygen

was absorbed in eight grams of oil when exposed to oxygen for 168 hours at 37°C (Table II). Practically the same amount of oxygen was absorbed in the oil within much shorter period of exposure at 37°C (Tables IV & V), the oil being previously contaminated with added fatty acids and moisture or traces of dissolved copper. The induction period of the oil, which was found to be 22.4 hours at 37°C, was reduced to practically zero at 100°C, to about one-fifth in presence of 0.0021 per cent dissolved copper and to about three-fourths on addition of free fatty acids and moisture (Table I).

As would be expected, the rates of destruction of vitamin A at 100°C was appreciably faster than that at 37°C. The presence of even traces of dissolved copper was found to exert a powerful catalytic influence on the destruction of vitamin A. The added fatty acids and moisture also hastened the destruction of vitamin A. About 72% loss of vitamin A occurred when the oil was exposed to oxygen at 37°C for 133 hours. Higher per cent of loss occurred within much shorter period of exposure at 37°C in presence of added fatty acids and moisture or traces of dissolved copper.

The added fatty acids and moisture hastened the development of peroxides also. The figures for peroxide number and loss of vitamin A as given in tables II and IV would generally indicate that the development of peroxide and the destruction of vitamin A would proceed simultaneously. At 100°C (Table III) or in presence of traces of dissolved copper (Table V), however, there was not much accumulation of peroxides in the oil, although the destruction of vitamin A was considerable. Rao (5) also observed that there was less peroxide formation in shark liver oil when either mixed with about 15 per cent copper or exposed to atmospheric air at higher temperatures.

These results indicate that the peroxide value of shark liver oil cannot always show conclusively the degree of deterioration produced in it. Peroxides can be proportional to deterioration only so long as they are formed at a greater rate than they are decomposed to the rancid products. If, however, the rate of decomposition of peroxides exceeds that of their formation, the peroxide-value decreases with increasing deterioration of the oil. It appears, therefore, some of the factors which hasten the rate of decomposition of peroxides in shark liver oil are the higher temperature of storage or the presence of copper-catalyst.

In almost all cases, the acid value increased slightly. The increase was comparatively higher at higher temperature or in presence of added fatty acids and moisture. In the presence of copper-catalyst, a slight fall in acidity was observed in the early stages of exposure.

The acetyl value also increased gradually along with the absorption of oxygen. The higher acetyl values in the oxidised samples might be partly due to the formation of any volatile or soluble fatty acids and partly due to the rise of acidity in the oil. Hence, if the corrections for any volatile or soluble fatty acids would be made, comparatively lower acetyl values might be obtained.

In each set of experiments, there was a steady decrease in iodine value with the absorption of oxygen. The rate of fall of iodine value, however, was not strictly proportional to the amount of oxygen absorbed. This may be explained by

supposing that numerous complex reactions may occur with the unsaturated acids and the oxygen absorbed. The hydrogen atoms necessary to complete some of these reactions may come from the other fatty radicals, thereby creating fresh centres of unsaturation that may again be oxidised.

In all the cases, saponification value did not alter in the early stages of oxidation. Towards the last stage only, a slight increase in the saponification value was observed.

#### SUMMARY

(1) The amount of oxygen absorbed at different stages of autoxidation in shark liver oil exposed to oxygen in thin layers was measured in an oxygen-absorption apparatus.

(2) The destruction of vitamin A and the changes of the important chemical characteristics in the autoxidised oils were studied under varying conditions viz., (a) at 37°C, (b) at 100°C, (c) on addition of fatty acids and moisture and (d) in presence of traces of dissolved copper.

(3) The induction period as well as the total period of exposure during which the maximum amount of oxygen was absorbed varied appreciably with the conditions of exposure, although the maximum amount of oxygen absorbed in the oil exposed under different conditions was practically of the same order.

(4) The development of peroxides at 100°C was lowest, although the destruction of vitamin A was considerable. The rate of formation of peroxides at 37°C in presence of traces of dissolved copper was much lower than that in absence of copper-catalyst, although the presence of copper hastened the destruction of vitamin A. The addition of free fatty acids and moisture hastened the development of peroxides as well as the destruction of vitamin A.

(5) In practically all the cases tried, it was found that along with the absorption of oxygen, the acid and acetyl values of the oil increased gradually and there was a steady fall of iodine value. Towards the last stage of oxidation, a slight increase in the saponification value was observed.

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LETTER TO THE EDITOR

HYPOGLYCEMIC ACTION OF ALLOXAN

SIR,

In experiments with rabbits we have presented evidences to show (1, 2) that the hypoglycemic action of alloxan is pancreatic in origin and is due to the release of insulin from the damaged islet cells. Carrasco-Formiguera (3) observed hypoglycemia after the intramesenteric injection of alloxan in dogs in which the pancreas was totally excluded from the circulation by clamping all the vessels of the pancreas. He is, therefore, of opinion that the hypoglycemia is extrapancreatic in origin and suggests that this is brought about by the direct action of alloxan on the liver. He, however, could not explain the absence of hypoglycemia after injection of alloxan in a rabbit made diabetic by a previous injection of alloxan (2). Houssay *et al.* (4) reported that totally depancreatized dogs injected with alloxan half an hour after the operation developed hypoglycemia. He, therefore, suggested that alloxan hypoglycemia is due to lack of glucose-formation by the liver after the injection of alloxan. He, however, gave no experimental evidence to prove his suggestion. In the present communication the glycogen content of the liver of rabbits, killed during hypoglycemic convulsions developed either after the injection of alloxan or insulin, has been presented.

Nine healthy Himalayan rabbits were allowed to take only water for 48 hours after which the animals were divided into three groups. Animals of one group, received intravenously a 10 per cent solution of alloxan, 200 mg. per kilo.; the second group of animals received a subcutaneous injection of 20 units of regular insulin while the third group of animals received no injection. The injected rabbits developed hypoglycemia 3 to 5 hours after the injection of alloxan or insulin. The animals had fits of hypoglycemic convulsions and when they were dying they were stunned by a blow on the head, the neck veins were cut and the liver was removed. The control animals were also similarly killed at the same time. Glycogen in the liver was estimated by the method of Grattan and Jensen (5). Blood sugar was estimated in the samples of blood taken before the injections and just before the animals were killed by the method of Hagedorn and Jensen (6). The results are given in the Table. The glycogen values are expressed as mg. of glycogen per 100 g. of liver and the blood sugar values are given as mg. per 100 cc. blood.



	Wt. of rabbit.	Blood sugar Before injection	After convulsion.	Liver glycogen.
Alloxan injected rabbits	1240 g. 1170 970	110 mg. % 121 117	24 mg. % 44 39	260 mg. % 140 980
Insulin injected rabbits	1130 920 970	87 114 119	30 35 21	580 420 1210
Control rabbits without injection	1080 780 920	110 105 98	— — —	1390 710 1100

The alloxan and insulin injected rabbits developed convulsions almost at the same time, the degree of hypoglycemia was similar and there was considerable decrease in the glycogen content of the livers. The results, therefore, indicate that alloxan hypoglycemia is not due to lack of glucose production in the liver as suggested by Houssay. As the symptoms developed in the rabbits injected with alloxan were similar to those of the insulin treated ones it may be suggested that the alloxan hypoglycemia in rabbit is pancreatic in origin.

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A. COMPARATIVE STUDY ON METABOLISM OF ISO-OLEIC AND  
SATURATED ACIDS OF HYDROGENATED GROUNDNUT OIL  
BY ALBINO-RATS IN RELATION TO AGE

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During recent years, a great deal of interest has centred round the nutritive value of Vanaspathi (hydrogenated fat). The most characteristic change in fatty acids composition during the catalytic hydrogenation of oils is the formation of iso-oleic acids. It is well established (1, 2) that during the first stages of hydrogenation of groundnut and other vegetable oils, the more unsaturated linoleic glycerides present are preferentially reduced to a mixture of oleic and iso-oleic glycerides and towards

the later stages a considerable amount of the iso-oleic glycerides formed is further converted into stearic derivatives. The nature and the extent of formation of iso-oleic acids may vary with the different conditions existing at the time of hydrogenation. Iso-oleic acid content of the various western brands of hydrogenated fats has been reported (1) to vary mostly from 5 to 25 per cent. In nine Indian brands of commercial Vanaspatis, iso-oleic acid content has been found (3) to vary from 1 to 23 per cent.

As iso-oleic acid is the only new constituent formed by hydrogenation, it is of great practical interest to obtain sufficient information regarding the metabolism of iso-oleic acid. Barbour (4) and Sinclair (5) have reported that iso-oleic acids are metabolized normally by the animal body and may be utilized as fuel as satisfactorily as the other fatty acid components of hydrogenated cotton seed oil. Spadola and Ellis (6) also have reported that ingestion of iso-oleic acid results in its deposition in the depot fat. Channon *et al.* (7) have observed that elaidic acid (a solid isomer of oleic acid) is treated normally in the body, appearing in liver, carcass and faecal fats in proportions dependent on its amount in the diet. Misra and Patwardhan (8) have found no significant difference between the digestibilities of Vanaspatis containing different proportions of iso-oleic acids. In the present communication, comparative studies have been made on the absorption, deposition and utilization of iso-oleic acids and saturated fatty acids of hydrogenated groundnut oil, both at low and high levels of intake, by albino-rats in relation to age.

#### EXPERIMENTAL

Although iso-oleic acids are unsaturated acids, they resemble saturated acids in their properties. They are solid at ordinary temperatures and form lead salts which are sparingly soluble in alcohol, so that in the lead-salt separation of mixed fatty acids, the major amounts of iso-oleic acids appear along with the saturated acids. The iso-oleic and the saturated acids were isolated from several pounds of a sample (A) of hydrogenated groundnut oil (M.P. 41°C), in batches of two pounds, by the lead salt separation method of Cocks *et al.* (9). The total amount of the mixtures of iso-oleic and the saturated acids thus isolated was melted and mixed uniformly with the original sample in suitable proportion so as to obtain a second sample (B) containing higher amounts of iso-oleic and saturated acids. The object of adding extra iso-oleic and saturated acids is to compare the metabolism of the total saturated and iso-oleic acids of hydrogenated groundnut oil in high and low concentrations. The iso-oleic and saturated acids content of the two samples are given in Table I.

Iso-oleic and saturated acid contents of dietary, body or faecal fat samples were estimated by the method developed by Cocks *et al.* (9) and recommended by Hilditch *et al.* (1). These authors only claim that this method gives results which are at least within 3.0 units per cent of the actual iso-oleic acids content. The amount of saturated acids tends to be somewhat lower, since minor amounts of lead myristate and palmitate pass into the unsaturated portion.

Sixty-four litters (32 males and 32 females) were weaned after 28 days when they weighed 36 to 44g. They were placed for four weeks on a fat-free synthetic diet composed of corn-starch (65%), extracted casein (15%), cane-sugar (10%), Osborne and Mendel (10) Salt Mixtures (5%) and Yeast powder (5%). They were then divided at random into two groups of 32 each (16 males and 16 females) and put for about twelve weeks on the experimental diet: The first group (A) receiving the diet which was similar to the previous diet with the difference that 30% starch was replaced by the same weight of Vanaspati (A) of low iso-oleic and saturated acids content and the casein content of the diet was raised from 15% to 30% at the expense of 15% starch; the second group (B) receiving the diet identical to that of the first group except that Vanaspati (B) of high iso-oleic and saturated acids content was used. The fat-free as well as the experimental diets were supplemented with vitamin A and D concentrates and vitamin E in propylene glycol (60 I.U. of vit. A, 10 I.U. of vit. D and 0.5 mg. of tocopherol acetate per rat per day). The protein content of the fat-supplemented diets was increased from 15% to 30%, as it has been well-established (II) that ingestion of high fat and low protein diet results in the production of dietary fatty livers, irrespective of the nature of the fat used.

Weekly records of weights and records of food eaten during the metabolism periods were maintained. When the rats completed four weeks on the experimental diet, the faeces were collected in groups of four of the same sex from eight animals of each series for a period of ten days, dried and analysed for fat by Soxhlet extraction with ether so as to determine the digestibility of the fats. The total faecal fat was extracted from the faeces collected from groups of four animals and analysed for iso-oleic and saturated acids content.

The eight animals of each series, which were used for the determination of digestibility and faecal fat composition, were anaesthetized with sodium amytal. The livers were dissected out, pooled in the same groups of four, weighed, dried and analysed for fat by Soxhlet extraction with ether. Per cent of liver-fat was calculated on the basis of wet weight of livers. The gastro-intestinal tracts were discarded, the combined bodies of the same groups of four rats were chopped into small pieces with a meat-chopper, finely minced into a pulpy mass in a meat-mincer, mixed uniformly and dried. A suitable aliquot was used for the determination of body-fat by Soxhlet extraction with ether. Per cent of body-fat was calculated on the basis of the wet weight of the body minus the gastro-intestinal tract and liver. The total body-fat was extracted from the rest of the dried carcass and analysed for iso-oleic and saturated acids content. The results obtained on rats when kept for four weeks on the experimental diet are recorded in Table II.

When the rats completed eight weeks on the experimental diet, the above sets of experiments were repeated in an identical way with the other eight animals from each series. The results are given in Table III.

Similarly, experiments on digestibility and faecal fat composition were carried out with the remaining sixteen animals of each series after keeping them for twelve

weeks on the experimental diet. Of these animals, eight rats (control) from each series were sacrificed for the determination of body-fat and deposition of iso-oleic and saturated acids as before. The results are shown in Table IV. The average weekly increase in weight over a period of twelve weeks is shown in Table IV A.

The remaining eight animals from each series were fasted for five days during which period, water only was given. During the fasting period, faeces from all the eight animals of each series were collected together to determine whether or not excretion of iso-oleic acid had taken place. At the end of the fasting period, the animals were weighed and sacrificed. The composite samples of four rat-bodies (excluding gastro-intestinal tract and liver) were minced, dried and analysed for body-fat as before. The total body fat extracted was analysed for iso-oleic and saturated acids content to determine whether these fatty acids had been depleted in proportion to the total fat-content. The results obtained are recorded in Tables V and VA.

### DISCUSSION

The average weekly increase in body-weight of rats is practically the same on the two dietary fats studied.

Although casein used was extracted thrice with alcohol, it was not possible to make the diet in bulk absolutely fat-free. The mixture of all the other ingredients excluding fat in the fat-supplemented diet was found to contain 0.31 per cent of ether-extractives. It was felt, however, that this low fat content of the diet will not affect the final results.

The digestibility of the fat has been calculated without subtracting the endogenous excretion of fat from the faecal fat. So the figures recorded give the apparent digestibility. In our previous experiments (12), however, it has been observed that the amount of endogenous excretion of fat in adult rats raised on fat-free diet is of the order of only 0.1 to 0.2 gms. per rat in five days. Hence, if the digestibility is calculated with the allowance for the presence of this small amount of endogenous fat, the figures for true digestibility will be only slightly higher than those recorded for apparent digestibility.

The digestibility of both the fat-samples appear to be slightly lower in rats completing four weeks on the experimental diet than when the same fats were continued for twelve weeks. No difference in digestibility, however, is noticed between the two periods of eight and twelve weeks on the experimental diet. In comparison with the digestibility of the original fat (A), that of the fat (B) with added saturated and iso-oleic acids appears to be slightly lower in all the three periods of study. No sex-difference in digestibility of both the fats is observed.

The saturated acids content of the faecal fat of rats of each group has been found to be higher than that of the corresponding dietary fat. Again, the saturated acids

TABLE I  
*Composition of dietary fats.*

Sample No.	Saturated acids per cent.	Iso-oleic acid per cent.
A	28.24	8.70
B	46.18	14.68

TABLE II

*Digestibility of fats, body and liver-fat contents and body and faecal fats composition of rats completing eight weeks on experimental diet. (Average values for four rats).*

Group.	Rat No. and Sex	Weight before sacrifice g.	Food intake in the metabolism period of 10 days g.	Fat intake in 10 days g.	Dry weight of faeces of 10 days g.	Faecal fat in 10 days g.	Digestibility per cent.	Per cent of faecal fat		Body fat per cent.	Per cent of body-fat.		Fresh liver wt. g.	Liver fat per cent
								Satd. acids.	Iso-oleic acids.		Satd. acids.	Iso-oleic acids.		
A	1 to 4 (M)	142.2	86.0	25.80	9.29	3.18	87.68	51.27	6.12	10.13	28.96	2.18	6.03	3.24
	1 to 4 (F)	119.1	78.1	23.43	8.67	3.07	86.90	53.13	6.23	11.02	29.21	2.05	5.12	3.08
B	1 to 4 (M)	141.0	74.5	22.35	10.02	3.81	82.94	76.10	10.14	10.45	29.64	5.81	5.98	3.01
	1 to 4 (F)	120.3	72.8	21.84	9.87	3.82	82.50	76.89	10.46	10.61	29.90	5.99	5.10	3.18

TABLE III

*Digestibility of fats, body and liver-fat contents and body and faecal fat composition of rats completing twelve weeks on experimental diet. (Average values for four rats).*

Group.	Rat No. and Sex.	Weight before sacrifice g.	Food intake in the metabolism period of 10 days g.	Fat intake in 10 days g.	Dry weight of faeces of 10 days g.	Faecal fat in 10 days g.	Digestibility per cent.	Per cent of faecal fat.			Per cent of body-fat.			Fresh liver wt. g.	Liver fat per cent.
								Satd. acids.	Iso-oleic acids.	Body fat per cent.	Satd. acids.	Iso-oleic acids.	body-fat.		
A	5 to 8 (M)	173.7	106.4	31.92	11.66	2.01	93.69	50.53	5.66	13.89	30.01	4.29	6.82	3.42	
	5 to 8 (F)	142.2	96.1	28.83	10.93	1.91	93.37	48.67	5.45	13.21	29.53	4.31	5.90	3.31	
B	5 to 8 (M)	174.9	104.0	31.20	13.45	4.31	86.18	75.31	8.94	12.92	30.80	9.00	6.90	3.38	
	5 to 8 (F)	143.5	92.3	27.69	12.87	3.82	86.22	72.70	8.65	12.68	30.19	9.13	5.82	3.50	

TABLE IV

*Digestibility of fats, body and liver-fat contents and body and faecal fat composition of rats completing four weeks on experimental diet. (Average values for four rats).*

Group.	Rat No. and Sex.	Weight before sacrifice g.	Food intake in the metabolism period of 10 days g.	Fat intake in 10 days g.	Dry weight of faeces of 10 days g.	Faecal fat in 10 days g.	Digestibility per cent.	Per cent of faecal fat.			Body fat per cent.	Per cent of body-fat.			Fresh liver wt. g.	Liver fat per cent.
								Satd. acids.	Iso-oleic acids.	Body fat per cent.	Satd. acids.	Iso-oleic acids.	body-fat.	body-fat.		
A	9 to 12 (M)	197.7	138.8	41.64	12.91	2.69	93.54	50.18	4.82	15.01	31.65	4.31	7.29	4.11		
	13 to 10 (M)	—	146.1	42.03	13.21	2.53	93.99	48.23	5.11	—	—	—	—	—	—	—
	9 to 12 (F)	161.8	120.4	36.12	12.61	2.44	93.24	49.44	5.01	13.90	33.83	4.43	6.61	3.58		
	13 to 16 (F)	—	115.6	34.68	12.02	2.29	93.40	52.01	4.93	—	—	—	—	—	—	—
B	9 to 12 (M)	198.1	130.7	39.21	14.89	5.38	86.28	76.25	8.80	13.88	32.28	9.09	7.34	3.46		
	13 to 16 (M)	—	133.0	39.90	15.21	5.42	86.42	73.71	8.69	—	—	—	—	—	—	—
	9 to 12 (F)	160.9	113.8	34.14	13.65	4.73	86.12	71.89	8.50	14.47	34.25	9.44	6.53	4.07		
	13 to 16 (F)	—	111.2	33.36	13.86	4.58	86.28	75.74	8.71	—	—	—	—	—	—	—

TABLE IV A

Gain in weight of rats over a period of twelve weeks (Average values of eight rats).

Group	Rat No. and Sex.	Weight after four weeks on fat-free diet g.	Weight after twelve weeks on experimental diet g.	Average weekly increase in weight g.
A	9-16 (M)	84.1	192.9	9.0 ± 0.40*
	9-16 (F)	77.5	156.9	6.6 ± 0.14
B	9-16 (M)	84.2	193.5	9.1 ± 0.32
	9-16 (F)	77.2	153.7	6.4 ± 0.27

\* Standard error of the mean.

TABLE V

Fat excretion during fasting and body-fat content and composition of body-fat after fasting for five days.

Group	Rat No. and Sex.	Weight* before fast- ing g.	Weight* after fasting g.	Body-fat* per cent.	Per cent of body-fat*		Dry wt. of total faeces of 8 rats during fasting. g.	Total faecal fat of 8 rats during fasting. g.	Total amount of iso-oleic acid in the faecal fat of 8 rats during fasting. g.
					Satd. acids.	Iso-oleic acids.			
A	13-16 (M)	203.6	154.4	6.41	40.93	2.92	8.31	2.03	0.13
	13-16 (F)	164.3	119.8	5.81	43.81	3.28			
B	13-16 (M)	202.0	155.4	6.03	43.50	6.31	8.62	2.11	0.24
	13-16 (F)	159.5	117.9	6.50	42.92	6.50			

\* Average values of four rats.



former case, the excess saturated acids may have been excreted in the fæces, thus accounting for the higher saturated acids content of the fæcal fat of rats of group B.

Iso-oleic acid content of the body-fat of rats completing four weeks on the experimental diet appears to be slightly lower than that of the body-fat of rats completing eight or twelve weeks on the same diet. The percentage of iso-oleic acids in the body-fat of rats raised on high iso-oleic diet has been found to be higher than the same in the body-fat of rats raised on low iso-oleic diet. It is evident from these results that rats are capable of absorbing iso-oleic acid and of depositing unchanged at least some of the absorbed acid in their depot-fat. Barbour (4), Spadola and Ellis (6), and Channon *et al.* (7) have recorded similar observations.

There is no significant difference in the percentage of fat content of liver with the two dietary fats studied ; nor does fatty liver result, the fat contents of the livers having been found to fall within the normal range.

With a view to indicate more clearly the rate of reduction of iso-oleic or saturated acids content of the body on fasting, the figures for iso-oleic and saturated acids contents of the body-fat of rats before and after fasting have been expressed as percentage of body-weight in Table VA. This sort of comparison is based on the usual assumption that the fasted rats contained before fasting the same level of fat as the controls of the same age, weight and sex and the composition of the body-fat of rats before fasting was identical with that of the body-fat of control rats. The results clearly show that as regards the utilization of the deposited fatty acids during fasting, the iso-oleic acids of the body are not inferior to the average body fatty acids. Rather it appears that they are depleted somewhat more readily than the average of the body fatty acids. This is in agreement with the observation of Barbour (4) and is particularly obvious when compared with the rate of depletion of saturated acids of the body on fasting. The somewhat larger concentration of saturated acids in the body after fasting may be owing to either the comparatively lower utilization of the body saturated acids or the preferential utilization of the body unsaturated acids or to both during fasting.

In accordance with the observations of Barbour (4), Iso-oleic acid has been found to be not concentrated in the fasting fæces, since the total amount of fæcal iso-oleic acid obtained from eight animals during the fasting period is only of the order of 0.1 to 0.2 gms., whereas approximately 8.2 gms. of iso-oleic acid was depleted from eight animals during the fasting period. This last figure is calculated from the data given in Tables V and VA, viz., an average rat weighing 203.6 gms. and containing 0.65 per cent iso-oleic acids in the body before fasting weighs 154.4 gms. and contains 0.19 per cent iso-oleic acid per body-weight after fasting.

#### SUMMARY

(1) Two groups of rats have been raised on synthetic diet supplemented with 30% fat in the form of either hydrogenated groundnut oil or the same with added

saturated and iso-oleic acids. The digestibility of the fats, extent of deposition of body and liver fats and faecal fat excretion with special reference to the absorption, deposition and utilization of iso-oleic and saturated acids have been determined at different ages of the growing rats.

(2) The average increase in body-weight of the two groups has been found to be practically the same. The digestibility of the fat containing higher amounts of saturated and iso-oleic acids appears to be slightly lower than that of the original sample. The digestibility of both the fats has been found to be somewhat lower in younger rats than in adult rats. No sex-difference in digestibility, deposition or excretion of the fats has been noted.

(3) The compositions of faecal fat samples of both the groups, when critically studied, indicate that the lowering of digestibility of the fat containing extra amounts of saturated and iso-oleic acids may be ascribed to the larger excretion of saturated acids, especially of the stearic and arachidic type, in the faeces. The composition of the faecal fat when compared with that of the corresponding dietary fat shows that the rate of excretion of iso-oleic acids is lower than that of the total saturated acids in both the groups.

(4) There is practically no difference between the body-fat contents or between the saturated acids contents of the body-fats of rats of the two groups. The percentage of body-fat has been found to be slightly lower in younger rats than in adult rats.

(5) The percentage of liver-fat has shown no significant difference with the two dietary fats and is normal.

(6) Iso-oleic acid has been found to be metabolised normally in the body. Iso-oleic acid has been found to be deposited in the body of rats approximately in proportion to the amount of iso-oleic acid present in the dietary fat. Iso-oleic acid content of the body-fat of younger rats appears to be slightly lower than that of the body-fat of adult rats.

(7) During fasting the deposited iso-oleic acids have been found to disappear more readily than the saturated acids or the average fatty acids of the body. Iso-oleic acid has been found to be not concentrated in the fasting faeces.

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STUDIES ON CHOLINE-ESTERASE PART VII. EFFECT OF DIFFERENT  
CHEMICALS ON THE ACTIVITY OF CRUDE AND PURE  
CHOLINE-ESTERASE

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Besides changes of temperature, hydrogen ion concentration and other physical factors, the activity of the enzyme can also be depressed by a variety of chemical reagents which are termed poisons with respect to the enzyme in question by previous workers.

A number of authors investigated the inhibitory effect of different reagents on the activity of choline-esterase present in the various body fluids of animals some of which are mentioned below. Corteggiani (1) found that 0.33% formol brought about a partial inactivation after being kept for several hours while 1% formol solution caused complete inhibition after 3-4 days. Kahane and Levy (2) studied

the inhibition brought about by the low concentration of drugs like eserine, geneserine, prostigmine, morphine, caffeine etc. The inhibition of choline-esterase in serum was also caused by the addition of morphine and apomorphine as observed by Bernheim and Berhheim (3). They suggested that the acetylcholine by the action of this drug might be accumulated in the brain. Jones and Tod (4) studied *in-vivo* the effect of eserine, pilocarpine and adrenaline with the result that the activity of the enzyme in the system was not in any way affected. Sobotka and Antopol (5) reduced the activity of the choline-esterase in serum with bile acids to different extents depending on the concentration of the acids and had also noticed that berberine got greater inhibitory effect than the former reagent.

Rothlin and Brugger (6) showed that the activity of the choline-esterase was inhibited by Ergotamine by 100-fold higher concentration than eserine. Keeser (7) found that glutathione, sympathol etc. activate choline-esterase activity while physostigmine, prostigmine, cocaine, hordenine and muscarine inhibited it. Glick and Antopol (8) inhibited choline-esterase activity by means of thiamine. Nachmansohn (9) showed that the inhibitory effect of hydroxyphenyl trimethyl ammonium bromide was intermediate between that of eserine and strychnine. Minot (10) compared the action of eserine and quinine in different concentrations on the serum choline-esterase. The activity of choline-esterase was largely but not completely lost by the chemicals like sodium oxalate, citrate, fluoride, arsenite and pyrophosphate while NaCNS had no inhibitory effect as shown by Massart and Dufait (11). Nachmansohn (12) further showed that the activity of the enzyme increased in presence of bivalent cations like Ba, Ca, Mg, Mn in minute concentration while the univalent cations also activated but in higher concentration.

Dufait and Massart (13) furnished evidence to the fact that choline-esterase was inhibited by sodium fluoride more powerfully on the acid side than on the alkaline side of the optimum  $pH$ . These authors further showed that dyes like methylene blue which had an alkaline reaction inactivated the choline-esterase activity and also that physostigmine when incubated with the enzyme beforehand inhibited the reaction rate more powerfully. The results of the experiments done with the different chemicals on choline-esterase in cobra venom would be interesting and are, therefore, recorded below. The purified enzyme choline-esterase was prepared by the procedure adopted by Chaudhuri (14).

## EXPERIMENTAL

### General Procedure

20 mg. of crude cobra venom for each of the four sets of experiments were taken in test tubes and dissolved in distilled water to each of which different amounts of the inhibitor were added. The volume was made upto 5 c.c. in each case. The  $pH$  of the solutions was adjusted as desired and the solutions kept at the room temperature ( $30^{\circ}C$ ) for a definite interval of time. When the scheduled time was over, the activity of the different solutions was determined at  $pH$  7.4 after deducting the respective control figures and the percentage of inhibition in every case as found out from the above data was enumerated in tables below. A control experiment containing the same amount of venom only without the inhibitor was kept side by side but no change in choline-esterase activity in any of the experiments was noticeable throughout the period.

TABLE I

*Silver Nitrate.*

Material and procedure.	Inhibition of the choline-esterase activity as per cent. of the original
1. 20 mg. of cobra venom + 0.3 cc. of N/10 AgNO <sub>3</sub> Sol.	17
2. 20 mg. of cobra venom + 0.5 cc. of N/10 AgNO <sub>3</sub> Sol.	28
3. 20 mg. of cobra venom + 0.8 cc. of N/10 AgNO <sub>3</sub> Sol.	42
4. 20 mg. of cobra venom + 1.0 cc. of N/10 AgNO <sub>3</sub> Sol.	49
The reaction was allowed to proceed for one hour at pH 7.4.	

TABLE II

...

*Potassium Ferricyanide.*

Material and Procedure.	Inhibition of the choline-esterase activity as per cent. of the original
1. 20 mg. of cobra venom + 0.3 cc. of N/10 K <sub>3</sub> Fe(CN) <sub>6</sub> Sol.	10
2. 20 mg. of cobra venom + 0.5 cc. of N/10 K <sub>3</sub> Fe(CN) <sub>6</sub> Sol.	17
3. 20 mg. of cobra venom + 0.8 cc. of N/10 K <sub>3</sub> Fe(CN) <sub>6</sub> Sol.	27
4. 20 mg. of cobra venom + 1.0 cc. of N/10 K <sub>3</sub> Fe(CN) <sub>6</sub> Sol.	30
The mixtures were allowed to react for twentyfour hours at pH 3.2 at a temperature of 8°C in the refrigerator under sterile condition.	

TABLE III

*Hydrogen Peroxide.*

Material and Procedure.	Inhibition of the choline-esterase activity as per cent. of the original
1. 20 mg. of cobra venom + 0.3 cc. of N/10 H <sub>2</sub> O <sub>2</sub> Sol.	14
2. 20 mg. of cobra venom + 0.5 cc. of N/10 H <sub>2</sub> O <sub>2</sub> Sol.	35
3. 20 mg. of cobra venom + 0.8 cc. of N/10 H <sub>2</sub> O <sub>2</sub> Sol.	48
4. 20 mg. of cobra venom + 1.0 cc. of N/10 H <sub>2</sub> O <sub>2</sub> Sol.	60
The reaction mixtures were adjusted at pH 7.4 and allowed to react for one hour at room temperature (30°C).	

TABLE IV

*Iodine.*

Material and Procedure.	Inhibition of the choline-esterase activity as per cent. of the original
1. 20 mg. of cobra venom + 0.3 cc. of N/20 I <sub>2</sub> Sol.	18
2. 20 mg. of cobra venom + 0.5 cc. of N/20 I <sub>2</sub> Sol.	35
3. 20 mg. of cobra venom + 0.8 cc. of N/20 I <sub>2</sub> Sol.	53
4. 20 mg. of cobra venom + 1.0 cc. of N/20 I <sub>2</sub> Sol.	65

The mixtures were adjusted at pH 7.4 and allowed to react for a period of one hour at room temperature (30°C).

TABLE V

*Sodium Iodoacetate.*

Material and Procedure.	Inhibition of the choline-esterase activity as per cent. of the original
1. 20 mg. of cobra venom + 0.3 c.c. of N/20 CH <sub>2</sub> ICOONa Sol.	20
2. 20 mg. of cobra venom + 0.5 c.c. of N/20 CH <sub>2</sub> ICOONa Sol.	40
3. 20 mg. of cobra venom + 0.8 c.c. of N/20 CH <sub>2</sub> ICOONa Sol.	56
4. 20 mg. of cobra venom + 1.0 c.c. of N/20 CH <sub>2</sub> ICOONa Sol.	69

Mixtures were adjusted at pH 7.4 and allowed to react for a period of one hour at room temperature (30°C).

TABLE VI

*Mercuric Chloride.*

Material and Procedure.	Inhibition of the choline-esterase activity as per cent. of the original
1. 20 mg. of cobra venom + 0.3 cc. of N/20 HgCl <sub>2</sub> Sol.	19
2. 20 mg. of cobra venom + 0.5 cc. of N/10 HgCl <sub>2</sub> Sol.	35
3. 20 mg. of cobra venom + 0.8 cc. of N/10 HgCl <sub>2</sub> Sol.	57
4. 20 mg. of cobra venom + 1.0 cc. of N/10 HgCl <sub>2</sub> Sol.	70

The reaction mixtures were kept for one hour at pH 6.0 at room temperature (30°C).

TABLE VII

*Sodium Fluoride.*

Material and Procedure.	Inhibition of the choline-esterase activity as per cent. of the original
1. 20 mg. of cobra venom + 0.3 cc. of N/10 NaF Sol.	21
2. 20 mg. of cobra venom + 0.5 cc. of N/10 NaF Sol.	35
3. 20 mg. of cobra venom + 0.8 cc. of N/10 NaF Sol.	53
4. 20 mg. of cobra venom + 1.0 cc. of N/10 NaF Sol.	67

The mixtures were allowed to react for one hour at pH 7.4 at room temperature (30°C).

*Effect of hydrogen sulphide, cysteine, ascorbic acid and sodium cyanide*

These chemical reagents have an inhibitory effect on some enzymes while on some others they have got just the opposite effect that is they increase the activity of the enzyme concerned. In the case of choline-esterase purified from cobra venom and that present in the crude venom, these reducing agents distinctly enhanced the activity to almost the same extent. Quantitative data regarding the behaviour of these activators are detailed below.

A. (i) 2 mg. of cobra venom + 2 cc. of 0.08 M Hydrogen sulphide sol. volume was made up to 5 cc.  
(ii) 5 mg. of pure choline-esterase + 0.5 cc. of 0.08 M Hydrogen sulphide volume was made up to 2.5 cc. pH of the solutions was adjusted at 6.0.

B. (i) 20 mg. of cobra venom + 2.0 cc. of M/10 cysteine sol.; volume was made up to 5 cc.  
(ii) 5 mg. of pure choline-esterase + 0.5 cc. of M/10 cysteine sol.; volume was made up to 2.5 cc.; pH of the solutions was adjusted at 4.2.

C. (i) 20 mg. of cobra venom + 2 cc. of M/10 ascorbic acid; volume was made up to 5 cc.  
(ii) 5 mg. of pure choline-esterase + 0.5 cc. of M/10 ascorbic acid; volume was made up to 2.5 cc.; pH of the solutions was adjusted at 3.2.

D. (i) 20 mg. of cobra venom + 2 cc. of M/10 sodium cyanide; volume was made up to 5 cc.  
(ii) 5 mg. of pure choline-esterase + 0.5 cc. of M/10 sodium cyanide; volume was made up to 2.5 cc.; pH of the solutions was adjusted at 3.2.

Controls of crude venom as well as of pure choline-esterase without the accelerators were kept side by side under similar conditions; and the results showed that choline-esterase activity was neither inhibited nor accelerated in the case of these controls during the experimental period.

The reaction mixtures were kept at room temperature (30°C) for a period of two hours. The activity of the different solutions was then determined as usual and expressed as per cent of the original. The results are tabulated below.



TABLE VIII

*Effect of reducing agents on cholin-esterase activity.*

Serial No.	Content of the choline-esterase activity as per cent. of the original.	
	Crude cobra venom.	Pure choline-esterase.
	(i)	(ii)
A	121	119
B	120	118
C	124	126
D	119	121

## SUMMARY AND DISCUSSION

Chemical reagents like iodine, monoiodoacetic acid, sodium fluoride etc. caused inhibition of choline-esterase to different extents, while others like cystein, hydrogen sulphide etc. got an activating effect on the enzyme. In which way the activation was brought about by these chemical agents has not yet been definitely established. When the activators are utilised to revive the latent portion of the activity, their function may be to transform the inactive form into the active form by a chemical process resulting in the manifestation of the increase in activity. In the case of choline-esterase the maximum accelerating effect was found to about 20% of the original. The phenomenon in this particular case may be explained to be due to the integrity of the thiol group assumed to be present in the enzyme molecule. A systematic study of the behaviour of these agents towards the groups responsible for the activity of the enzyme are represented in the Part VIII of this series.

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STUDIES ON CHOLINE-ESTERASE PART VIII. REVERSIBLE  
INACTIVATION OF CHOLINE-ESTERASE

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Of all the controlling factors responsible for the activity of the enzyme, the influence of hydrogen-ion-concentration is very great as shown by Sorensen (1) in a series of classical papers. It is, therefore, necessary that the nature of the activity- $pH$  curve should be known first of all. Besides, the changes in hydrogen-ion-concentration, the activity of the enzyme is liable to be changed by various

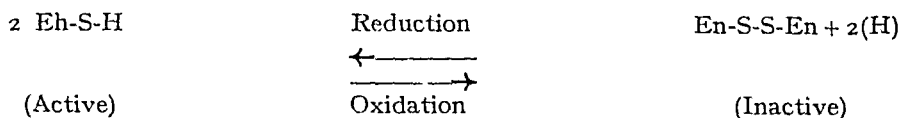
"salt-effects." Activation of the enzyme with respect to a reagent has been interpreted by various authors in the light of co-enzyme action, salt-effect, changes in oxidation-reduction state of their specific activators, removal of inhibitory metal ions etc. each of which rests upon a reasonable and strong hypothesis coupled with experimental evidences.

It is known that metallic salts like silver nitrate, mercuric chloride etc., had a depressing effect on the activity of the enzymes in general. But the inhibition in the case saccharase caused by silver ions was fully restored by the action of hydrogen sulphide as shown by Euler and Svanberg (2). This effect was explained by Myrback (3) to be a dissociable silver salt of the amphoteric enzyme. The inhibitory action of some metallic salts on the enzyme papain with the subsequent regeneration of its activity by some chemical reagent was interpreted by Krebs (4) as the removal of the inhibitory metal ions. Further, it was observed by Mendel and Blood (5) that the papain activity was enhanced in presence of HCN and H<sub>2</sub>S. Grassmann *et al* (6) showed that the activities of papain and Kathepsin was accelerated by organic sulphydryl compounds such as glutathione and cysteine. Hellerman and Perkins (7) while studying the inhibition of the enzyme papain with the subsequent activation showed the same thing to occur in the case of crystalline urease. It was also noted by Perlzweig (8) that the weakening of the nitroprusside test for the sulphydryl groups during aeration resulted in the partial inactivation of the crystalline urease. Summer and Poland (9) showed that although sulphydryl compounds like glutathione might be present in jack bean meal yet crystalline urease which was completely free from glutathione etc. responded to nitroprusside test.

Hellerman, Perkins and Clark (10) thought that the phenomena associated with the inactivation and reactivation of the enzyme might be attributed to the oxidation and reduction of the substituent sulphydryl groups of the enzyme itself but not to the changes in the supplementary activators.

According to Bersin (11) "the active enzyme is thus to be looked upon as a thiol compound while its inactive form is disulphide compound".

This may be represented as follows:



Hopkins *et al* (12) showed that the major portion of the activity at least, if not all, of the enzyme succinic dehydrogenase was dependent upon the integrity of the thiol group; as the enzyme activity was completely inhibited when exposed to the action of the oxidised glutathione simultaneously with the disappearance of the sulphydryl group but when the activity was fully restored under the influence of reduced glutathione, the sulphydryl group also reappeared at the same time. Avery and Neil (13) working with pneumococcal haemolysin inactivated it by treatment with air and hydrogen peroxide while Neil (14) subsequently restored the activity with the help of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> or with certain anaerobic bacteria. Schwachmann *et al* (15) inactivated haemolysin extract by suitable oxidising and mercaptide-forming reagents like cuprous oxide and certain organomercurials. This inacti-

vated haemolysin produced by these agents was reactivated by many thiol compounds and by some reducing agents like hydrogen cyanide, ascorbic acid etc.

Nachmansohn and Lederer (16) had shown that choline-esterase prepared from the electric organ of the *Torpedo Vulgaris* was inactivated through prolonged dialysis against distilled water, but its activity was found to be restored by means of reducing agents such as glutathione. It had also been shown by them that when the same enzyme preparation was partly inhibited by iodine, the activity was restored by the addition of glutathione from which they suggested that at least a part of the activity of the enzyme depended upon the integrity of the sulphydryl group.

With the idea in mind that choline-esterase purified from cobra venom might behave in the same way as that prepared from other sources the enzyme was subjected firstly to undergo partial inactivation by means of different oxidising agents like those mentioned below followed by subsequent treatment with reducing agents like cysteine, hydrogen sulphide, ascorbic acid and sodium cyanide. Regeneration of the activity was possible as will be evident from the following experiments. The characteristic of choline-esterase was that once the activity was completely lost it was not possible to restore it again. The pure enzyme choline-esterase used in the following experiments was purified by the procedure described by Chaudhuri (17).

## EXPERIMENTAL

### *General Procedure*

The general procedure adopted for finding out the reversible inactivation of the enzyme choline-esterase may be stated as follows:

25 mg. of pure choline-esterase isolated from cobra venom was taken and dissolved in redistilled water to which a requisite amount of the inhibitory agent, as for example, Iodine was added the volume being made upto 10 c.c. with redistilled water. The  $pH$  of the reaction mixture was adjusted as desired. It was then kept at the room temperature ( $30^{\circ}C$ ) for a definite period after which the extent of inhibition was determined from a portion of the reacting mixture. At the same time, 5 mg. portion of this partly inactivated enzyme was taken in each of the four different test tubes to which a fixed amount of the four reducing agents mentioned above were added, the volume being made upto 5.0 cc. in each case. The mixtures in the four test tubes were adjusted to the required  $pH$ . After a certain time by which the reaction was over, the activity of the enzyme was measured in the usual way at  $pH$  7.4. It may be mentioned here that the reducing agents at the concentrations used had some activating effect on the pure enzyme or the enzyme present in crude venom as had been shown in experiments in Part VII of this thesis, the maximum increase in activity being 20% of the original in the case of cysteine, hydrogen sulphide, ascorbic acid and sodium cyanide. If activators were used in higher concentrations, no further increase in activity was noticeable. Control experiments for each of the experiments were kept side by side but the change in activity of choline-esterase was not noticed in each case. The results are recorded in tables below:

TABLE V

*p*-benzoquinone and activators.

Material and Procedure.	Content of choline-esterase activity as percentage of the original.
A. 25 mg. of pure choline-esterase + 0.5 cc. of M/10 <i>p</i> -benzoquinone— <i>p</i> H 7.4 kept for one hour at the room temperature (30°C)	39
1. 5 mg. portion of (A) + 0.5 cc. of 0.08 M hydrogen sulphide— <i>p</i> H 6.0	122
2. 5 mg. portion of (A) + 0.5 cc. of M/10 cysteine— <i>p</i> H 3.2	123
3. 5 mg. portion of (A) + 0.5 cc. of M/10 ascorbic acid— <i>p</i> H 3.2	119
4. 5 mg. portion of (A) + 0.5 cc. of M/20 sodium cyanide— <i>p</i> H 3.2	110

The reaction solutions were kept for a period of two hours at the room temperature (30°C).

TABLE VI

*Potassium ferricyanide and activators.*

Material and Procedure.	Content of choline-esterase activity as percentage of the original.
A. 25 mg. of pure choline-esterase + 0.5 cc. of N/20 potassium ferricyanide— <i>p</i> H 3.2 ; kept at 8°C in the frigidaire in the sterile condition for a period of 24 hours	50
1. 5 mg. portion of (A) + 0.5 cc. of 0.8 M hydrogen sulphide— <i>p</i> H 6.0	118
2. 5 mg. portion of (A) + 0.5 cc. of M/10 cysteine— <i>p</i> H 4.2	124
3. 5 mg. portion of (A) + 0.5 cc. of M/10 ascorbic acid— <i>p</i> H 3.2	116
4. 5 mg. portion of (A) + 0.5 cc. of M/10 sodium cyanide— <i>p</i> H 3.2	109

The reaction mixtures were kept at the room temperature (30°C) for a period of two hours.

# SUMMARY AND DISCUSSION

Partial inhibition of the enzyme choline-esterase brought about by various mild oxidising agents like those used above were more than fully restored by means of cysteine, hydrogen sulphide, ascorbic acid and sodium cyanide under the conditions detailed above. Now in this part of the work, proof was put forward in the way of the reversible inactivation of the enzyme brought about by a number of specific reagents which played an important role in oxidising as well as in reducing the thiol unit associated with the choline-esterase molecule. Compounds of the type  $RH_2$ ,  $R$  which are unable to react with the thiol compound were also seen to have no effect on the activity of this enzyme.

Further an increase of about 20% of the activity of this enzyme was noticeable when it was kept in contact either with cysteine, hydrogen sulphide, ascorbic acid or sodium cyanide for a definite period. This phenomenon may be explained by supposing that a part of the enzyme existed in an inactive form due to its disulphide configuration. After treatment with the reducing agents to convert it into a sulphydryl state, the increased activity was observed. From this point of view it may be said that a part of the activity at least may depend upon the integrity of the thiol compound present in the enzyme.

Another way of looking at the sulphydryl problem in protein molecule is based upon the theory of denaturation as suggested by Neurath (17). He put forward the view that upon denaturation of the protein the sulphydryl group is open to be attacked by the specific reagents and the consequent destruction of its biological characteristics. On the basis of this theory reactivation of the inactivated enzyme choline-esterase would involve two separate processes (1) the regeneration of the sulphydryl group and (2) the reversal of denaturation of the protein molecule. The heavy metal ions are known to cause denaturation of proteins but how far this process is reversible remains yet to be ascertained in the majority of cases.

## ACKNOWLEDGEMENT

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### A. Casein Hydrolysate Basal Medium.

*Casein Sample:* A locally made casein having 12.8 per cent nitrogen was used.

## EXPERIMENTAL

### *Methods of hydrolysis:*

- (1) The method of combined sulphuric and hydrochloric acid digestion was utilised, the quantity of HCl being so adjusted as to give 0.5 to 0.6 per cent of chloride (NaCl) after neutralisation with NaOH in the final base. The mixture was heated initially over a water-bath for 1 hour and then refluxed over an electric bath consecutively for varying periods viz., 71, 6, 12, 24, 48, 72, 96, 120, 144, 168, 192, and 216 hours to find out at what stage the medium could be made free from an appreciable amount of precipitable protein at 33, 50 and 100 per cent saturation of  $\text{Na}_2\text{SO}_4$ . For neutralising  $\text{H}_2\text{SO}_4$  a cheap commercial variety of  $\text{BaCO}_3$  was found as good as the costly  $\text{Ba(OH)}_2 \cdot 8\text{H}_2\text{O}$  which was practically unavailable at the time of experiment due to the prevailing war conditions. The neutralised filtrate was clarified by treatment with  $\frac{1}{4}$  per cent "Norit" charcoal. The pH was adjusted between 7.4 and 7.6 by NaOH and the nitrogen content was determined by microKjeldahl technique.
- (2) The method of White and Elman (8) for digesting casein only with 2.6  $\text{NH}_4\text{SO}_4$  acid was also used. The rest of the procedure after hydrolysis was the same as in the case of combined acid hydrolysis except that the required quantity of NaCl had to be added separately to bring its concentration to 0.5 per cent in the final medium.

### *Nitrogen Recovery at Different Periods of Digestion:*

The rate and amount of hydrolysis, as also the recovery of nitrogen, depended not only on the period of digestion and the nature, quantity, and normality of the acid or acids used but also upon the arrangement of heating. It was noted that direct heating by a gas flame over a wire gauge was better than heating in a water bath, the latter being a little better than an ordinary electric stove. An interrupted heating arrangement did not, however, materially affect the final product but for a large scale production steam heating arrangement would be preferable.\*

By the combined acid mixture method the maximum recovery of total nitrogen was 97.6 per cent after 72 hours of digestion as against 79.2 per cent by the other process. Beyond this period there was slight reduction of total nitrogen recovered due to partial disintegration into ammonia. The amount of nitrogen lost due to neutralisation and clarification varied between 10 to 16 per cent. It was least

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\*Such a steam-heating arrangement in a large glass-lined kettles has since been installed at the Haffkine Institute, Bombay. The temperature of boiling with either process mentioned above was about 99.5°C but later, with the acid mixture slightly modified by Sokhey it adjusted itself to 101.5°C allowing the effective period of digestion to be reduced by 24 hours.

(10 per cent) when the  $\text{BaSO}_4$  precipitate was washed on the Buchner's funnel with hot water.

*Amount of Precipitable, Amino and Ammonia Nitrogen in Relation to the Periods of Digestion.*

Since a protein-free medium was what was aimed at the amount of nitrogen precipitable at one-third, one-half, two-thirds and full saturation of  $\text{Na}_2\text{SO}_4$  was estimated for various samples, each of which was also tested for amino-N in the minimum of time of digestion. The results are given in tables I and II.

TABLE I

*Amount of precipitable nitrogen, amino and ammonia nitrogen expressed in percentage of total N, in relation to the period of digestion of casein with the combined  $\text{H}_2\text{SO}_4$  and  $\text{HCl}$  acid mixture.*

Period of digestion (in hours)	$\text{Na}_2\text{SO}_4$ concentration				Amino-N (including $\text{NH}_3$ )	Precipitable N Crude broth.	Ammonia
	33%	50%	66%	100%			
1	15.80	22.80	27.20	45.30	—	—	—
6	9.10	11.40	15.20	18.90	34.20	19.50	—
12	6.90	9.30	10.80	13.00	41.50	13.20	—
24	5.01	6.70	6.84	7.80	46.70	7.20	1.6
48	1.80	3.24	3.36	3.82	57.10	3.50	2.3
72	0.28	0.80	1.30	2.10	63.30	1.90	3.5
96	0.20	0.52	0.70	0.84	64.90	1.01	4.0
120	Nil	0.26	0.60	0.60	66.40	0.70	6.6
144	"	Nil	Negligible	0.40	67.50	0.45	7.6
168	"	"	"	0.30	65.80	0.30	6.3
192	"	"	"	0.26	66.60	0.30	6.9

TABLE II

*Amount of precipitable, amino and ammonia nitrogen expressed as percentage of total nitrogen according to the period of digestion with T.P.  $\text{N-H}_2\text{SO}_4$ .*

Period of digestion (in hours)	Heating arrangement.	$\text{Na}_2\text{SO}_4$ concentration				Amino-N (including $\text{NH}_3$ )	Ammonia N percentage of NPN after neutralisation and clarification.
		33%	50%	66%	100%		
6	Over wire gauge and direct flame.	8.8	10.8	14.0	18.0	39.0	0.5
6	Over wire gauge.	9.3	12.7	15.4	19.5	33.0	0.18
6	Over electric stove	9.6	12.0	15.7	20.2	32.3	0.4
12	"	7.4	9.8	11.0	13.0	40.8	0.02
24	"	5.8	7.5	8.0	8.8	45.7	1.5
48	"	2.3	3.7	3.0	4.5	55.1	2.2



It will be seen from table I that the amount of amino-N increased progressively till 72 hours and thereafter, the progress slowed down and remained stationary after 120 hours (see chart I), the highest figure, 67.5 per cent, being reached after 144 hours of digestion. When the period of digestion is further increased there is certain amount of disintegration of amino-acids as indicated by the increase in the yield of  $\text{NH}_3\text{-N}$ . On the whole, there is no practical gain in the total amino-nitrogen after 72 hours. On the other hand, the amount of precipitable nitrogen which has a special bearing on the isolation of the specific protein substance of *P. pestis*, diminished progressively but it could not be completely removed even after 192 hours of digestion. At 33 per cent level of  $\text{Na}_2\text{SO}_4$  concentration there was no precipitable nitrogen after 120 hours and it was only  $\frac{1}{4}$  per cent after 72 hours. At 50 per cent level it was not recoverable after 144 hours and was roughly  $\frac{1}{4}$  per cent after 120 hours,  $\frac{1}{2}$  per cent after 96 hours and 0.8 per cent after 72 hours of digestion. With full  $\text{Na}_2\text{SO}_4$  saturation of 144, 120, 96 and 72 hours samples the figures are 0.4, 0.6, 0.84 and 2.1 per cent respectively (see chart II).

On comparison of the figures given in table I with those in table II, it is seen that at all periods of digestion the amount of precipitable nitrogen is higher and amino-nitrogen slightly lower with 2.6 N  $\text{H}_2\text{SO}_4$  digestion than with the combined  $\text{HCl} + \text{H}_2\text{SO}_4$  mixture. Thus, the latter method was preferred for the preparation of crude casein hydrolysate.

#### *Chloride content of the hydrolysate:*

In the  $\text{H}_2\text{SO}_4 + \text{HCl}$  mixture the quantity of  $\text{HCl}$  was so adjusted that after neutralisation with  $\text{NaOH}$ , the  $\text{NaCl}$  content remained round about 0.5 per cent in the final medium containing 2.4 to 2.7 mg. of nitrogen/ml. The actual  $\text{NaCl}$  content of the final media prepared from the bases subjected to different periods of digestion ranged between 0.52 to 0.62 per cent.

#### *Tryptophane content of the hydrolysate:*

It is known that tryptophane, one of the amino-acids in the composition of casein, is destroyed during acid hydrolysis. Fortunately, this amino-acid though essential for the growth of the organisms like *Bact. typhosum* was not so for *Past. pestis*. Rao (9) also came to the same conclusion. However, White and Elman (8), in a preliminary work, suggested that hydrolysis with 2.6 N  $\text{H}_2\text{SO}_4$  for 6 hours would liberate 60 per cent of amino-N and retain about 85 per cent of the tryptophane content, as tested by the method of Folin and Ciocalteu (10). In order that the medium could be more universally used several samples were tested after various periods of digestion by the above method as well as by the recent methods of Eckert (11). The former gave a much higher reading than the latter which, however, gave more consistent result when compared against known controls. The results obtained at different periods of digestion are given in table III.

CHART I.

PERCENTAGE OF AMINO-N RECOVERED AT DIFFERENT PERIODS OF ACID - DIGESTION OF CASEIN

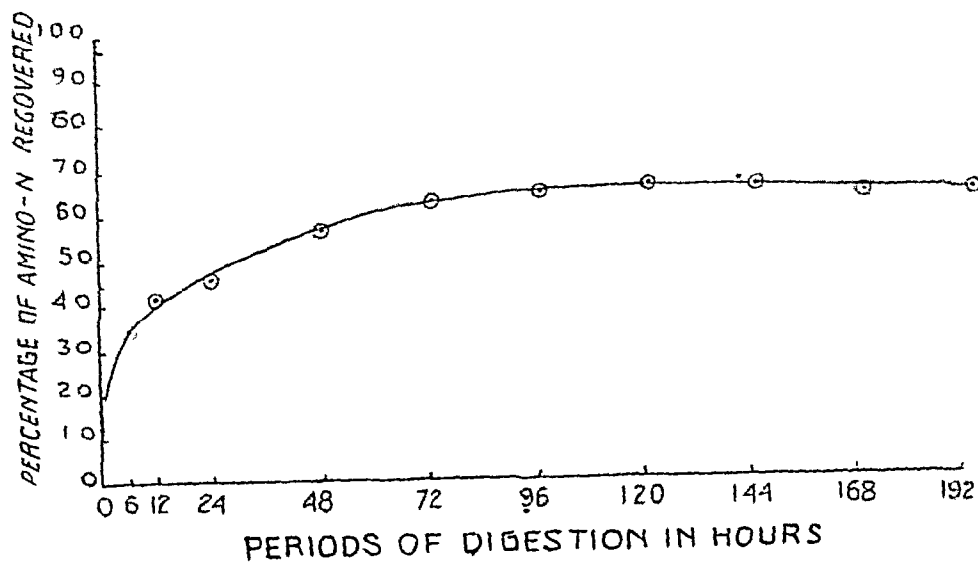


CHART II.

PERCENTAGE YIELD OF PRECIPITABLE N AT DIFFERENT  $\text{Na}_2\text{SO}_4$  CONCENTRATION IN RELATION TO THE PERIODS OF DIGESTION

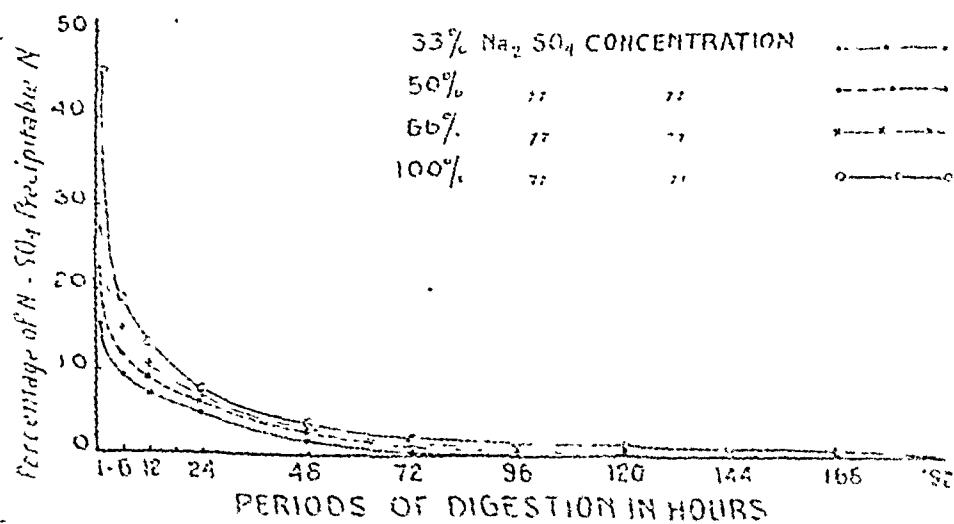


TABLE III

*Percentage of tryptophane recovered after different periods of digestion of casein with 2.6 N H<sub>2</sub>SO<sub>4</sub>.*

	Period of digestion in hours.					
	6	9	12	18	24	48
Range of percentage of tryptophane recovered.	40-45.8	30-35	28-31	20.3-24.1	10.2-11.0	7.3-8.0

The results in table II show that only 40-45 percent of tryptophane is retained after 6 hours digestion of casein with 2.6 N H<sub>2</sub>SO<sub>4</sub> and only 7 to 8 percent after 48 hours, whereas the digestion carried out with H<sub>2</sub>SO<sub>4</sub> and HCl mixture for 72 hours left only 1.1 to 1.5 percent of tryptophane.

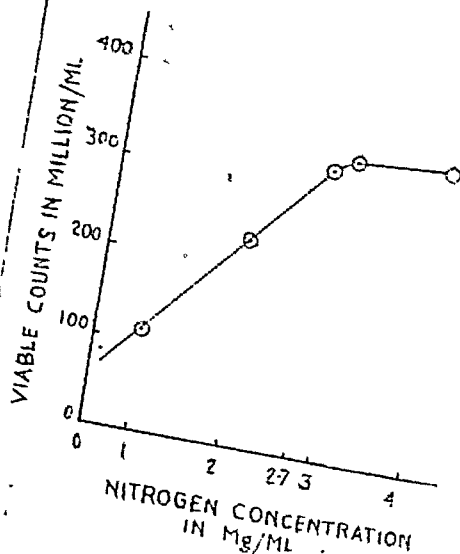
*Optimum nitrogen value of casein hydrolysate:*

The average nitrogen value of the acid-digested mutton broth in which the Haffkine plague vaccine was being prepared was 2.3 to 2.5 per ml. To determine the optimum nitrogen concentration required for a good growth of *P. pestis* in casein hydrolysate broth the nitrogen content was varied between 1 to 4 mg/ml keeping the other constituents undisturbed and the media were tested for 48 hours viable count. The result is shown in chart IV. It will be seen that there was a gradual rise of the viable count from 1 mg. to 4 mg. N samples but the curve flattened out beyond 3 mg N/ml, and the difference between 2.7 mg and 3 mg N/ml was not very significant. Since the NaCl content could be automatically adjusted to 0.5 percent at 2.7 mg. level of nitrogen per ml, this was taken as the optimum for the final medium in the succeeding experiments.

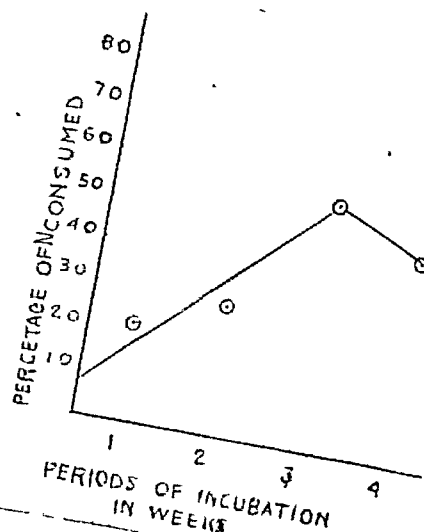
In order to find out whether the above concentration of nitrogen would be sufficient for 4 weeks' growth, flasks containing media were inoculated with *P. pestis* and incubated at 28°C for 1, 2, 3 and 4 weeks respectively, removed at the end of each interval and the nitrogen consumed was calculated as follows: The growth in each flask was centrifuged off and the supernatant clarified through L<sub>2</sub> candle. The nitrogen per ml of the filtrate was estimated and the loss due to bacterial growth ascertained. The filtrate was then precipitated with Na<sub>2</sub>SO<sub>4</sub>, precipitate recovered, dialysed, dried and weighed (to the constant weight). From this the nitrogen lost due to the formation of specific soluble substance was estimated. The total nitrogen loss due to bacterial growth and specific substances for different periods of incubation was found to be as follows: At the end of 1 week—0.58 mg., at the end of 2 weeks—0.78 mg., at the end of 3 weeks 1.5 mg. and at the end of 4 weeks—1.3 mg, i.e., 21.5, 28, 55.5 and 48 per cent respectively of the total nitrogen content. (Chart V). The maximum quantity of nitrogen used up was, therefore, only 55.5 per cent or 2.7 mg N/ml. available in the medium. Thus from this point of view also the quantity of nitrogen was considered sufficient.

# CASEIN HYDROLYSATE MEDIUM FOR P. PESTIS

**CHART IV**  
AVERAGE 48 HOURS VIABLE COUNTS  
IN MILLION/ML. IN RELATION TO N CONCENTRATION  
OF CASEIN HYDROLYSATE BROTH



**CHART V**  
PERCENTAGE CONSUMPTION OF CASEIN HYDROLYSATE  
BROTH IN RELATION TO THE PERIODS  
OF INCUBATION



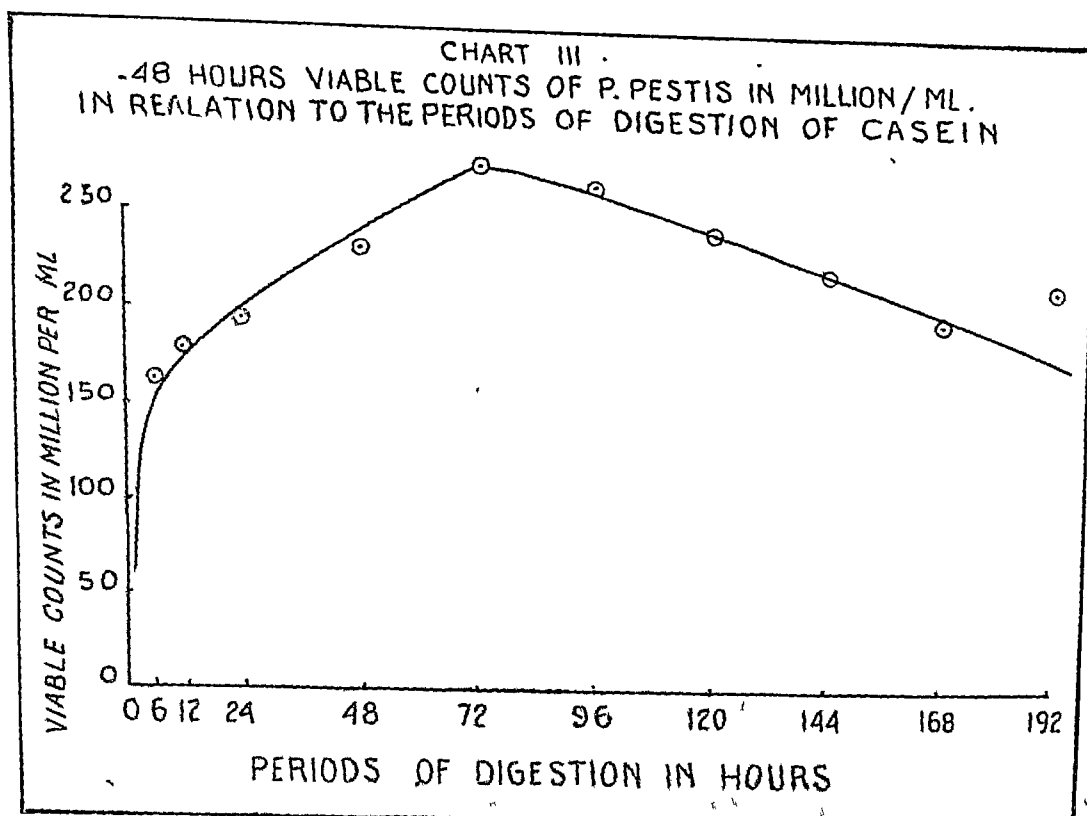
## Growth of *P. pestis* in different samples of crude casein hydrolysate:

Since one of the most important criteria of a good medium is the amount of growth, all broth samples made out of the hydrolysates of different periods of digestion, were tested for viable counts by the standard method developed by Sokhey (12). The nitrogen content of each sample was adjusted to 2.7 mg. N/ml. and the pH to 7.4 with NaOH. The media were sterilised at 110°C for 10 minutes in the autoclave, inoculated with virulent *P. pestis* and the viable counts made after 48 hours' incubation at 28°C. The results are given in table IV.

**TABLE IV**  
48 hours viable counts of virulent *P. pestis* in different samples of  
hydrolysate broth (base).

Periods of digestion (hrs.)	Viable counts in million per ml.	Periods of digestion (hrs.)	Viable count in million per ml.
1	58 (average of 3 tests)	60	265 (Average of 3 tests)
6	162 (average of 7 tests)	120	230
12	180 (average of 3 tests)	144	220
21	195 (average of 3 tests)	—	—
48	231 (average of tablets)	168	196 (Average of 3 tests)
72	273 (average of 5 tests)	192	210

It will be seen from table IV that the viable counts of virulent *P. pestis* increased gradually up to 72 hours digestion and did not change much till about 120 hours. Thereafter, the count showed a definite tendency towards a fall (see also Chart III) Thus 72 hours digest was tentatively chosen for further studies.



#### Phosphorus content :

The  $\text{PO}_4$  content of the nutrient and acid-digested mutton broths which were generally used for the growth of *P. pestis* were 0.52 mg and 0.23 mg/ml respectively, while that of crude casein hydrolysate was only 0.129 mg/ml. Thus, it was highest in nutrient broth and lowest in casein hydrolysate. The  $\text{PO}_4$  concentration was, therefore, raised to 0.38 mg./ml, a level intermediate between the other two broths, by adding anhydrous  $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  to the medium in proportion of 0.4 mg and 0.13 mg/ml. The  $\text{pH}$  was adjusted to 7.4-7.6 by  $\text{NaOH}$  and the medium made iron-free by means of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  according to the method described by Mueller (13), so that the requirement of iron, if any, could be experimentally determined. Twenty one batches of 72 hours digest of casein hydrolysate prepared in the above way gave 48 hours viable count ranging between 240 and 294 million per ml, the majority giving readings above 300 million and the average being 320 million per ml.

The various nitrogen distribution of the three media, viz., acid-digested mutton broth, gelatin and casein hydrolysates, the last two being practically protein-free, have been analysed and the results given in table V for comparison of viable counts for comparison.

*Nitrogen distribution of acid-digested mutton broth, gelatin and casein hydrolysates.*

Contents	Acid-digested mutton broth	Gelatin hydrolysate	Casein hydrolysate
1. Total N mg/ml.	2.616 mg	2.531 mg	2.672 mg
2. Amino-N mg/ml.	0.66 mg (25.4%)	0.847 mg (33.4%)	1.7 mg (64.7%)
3. Ammonia-N mg/ml.	0.26 mg (10%)	0.0272 mg (1.07%)	0.126 mg (4.7%)
4. Non-protein N mg/ml.	1.04 mg (40%)	2.361 mg (93.2%)	2.10 mg (82%)
5. Tungstate-pptble protein N mg/ml.	1.576 mg (60%)	0.17 mg (6.6%)	0.462 mg (17.2%)
6. 50% Na <sub>2</sub> SO <sub>4</sub> pptble N mg/ml.	0.136 mg (5.2%)	Negligible.	0.213 mg (8.0%)
7. Na <sub>2</sub> SO <sub>4</sub> -full saturation pptable N mg/ml.	0.625 (23.9%)	0.13 mg (5.1%)	0.056 mg (2.1%)
8. Average 48 hours viable count/ml.	150 million	160 million	320 million
9. Biuret reaction.	+	—	±

The acid digested mutton broth contained a heavy amount (approx. 60%) of tungstate-precipitable nitrogen of which about 24 percent was constituted by Na<sub>2</sub>SO<sub>4</sub> precipitable nitrogen. The non-protein nitrogen in the medium was about 40 percent, the amino-N contributing only 25.4 percent and ammonia-N 10 percent. Thus, quite a considerable amount of nitrogen remained as either polypeptides or still less broken up protein molecules. The gelatine hydrolysate medium\* on the other hand, had the highest amount (93.2%) of non-protein nitrogen and the least amount 1.07% of ammonia-N and only negligible amount of precipitable nitrogen at 50 percent Na<sub>2</sub>SO<sub>4</sub>, although the amino-N was only about 33.4 percent. The corresponding figures for the casein hydrolysate medium were 82, 4.7, 0.8 and 64.7 percent respectively. The precipitable nitrogen at full saturation of Na<sub>2</sub>SO<sub>4</sub> was 2.1 percent in the latter as against 5.1 percent in the gelatin hydrolysate. The comparatively low amino-N content indicate that a considerable amount of protein molecules remained as peptone and was only partially converted into amino-acids by the process of digestion. The casein hydrolysate medium in this respect had therefore an advantage over the gelatin hydrolysate. The average 48 hours viable count in this base medium was 160 million per ml as against 320 million per ml. in the casein hydrolysate and 150 million per ml in the acid-digested mutton broth. It may, however, be mentioned here that the average 48 hours viable count of *P. pestis* in nutrient broth was 400 million per ml thus indicating that nutritionally 72 hours casein digest needed further improvement.

\* The medium was prepared as follows: 100 g. of gelatin (Merck's) was taken up in 100 ml. of conc. H<sub>2</sub>SO<sub>4</sub> (Merck's) and 900 ml. of distilled water and autoclaved at 120°C (Rao used 128°C) at 20 lbs. pressure for 1½ hour in a pyrex flask and then neutralised by baryta (avoiding excess); to 1 litre of this clear golden yellow hydrolysate broth containing 2.5 mg. of N/ml. 5 g. of NaCl, 0.5 g. of Na<sub>2</sub>HPO<sub>4</sub>, 2H<sub>2</sub>O, 0.5 g. of MgSO<sub>4</sub> (dry) and 100 mg. of l-cystine (separately dissolved in a few ml. of N HCl) were added and sterilised. (Sometimes a precipitate of Mg<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> appeared which was filtered off).

## B. NUTRITIVE SUPPLEMENT OF THE BASAL CASEIN HYDROLYSATE BROTH.

The main purpose of this study was to evolve a protein-free medium which would give the best yield of the specific protein substance of plague bacillus in more or less pure form, the potency of which naturally depended upon the character of the antigenic quality of the growth of the organism in the medium. But as could be seen from above that as far as the growth is concerned the casein hydrolysate basal medium did not come up to the expected standard compared to the efficiency of nutrient broth. Attempts were, therefore, made to improve the medium by the addition of certain nutritive supplement. Rao (9) found proline, phenylalanine and cystine as the three essential amino-acids for the growth of *P. pestis*. He (14) also noted that some of the complex compounds like haematin, cozymase, thiamin and nicotinic acid more or less stimulated the growth in a chemically defined medium. Recently, Duodoroff (15) in a similar study obtained heavy growth of *P. pestis* in a synthetic medium with glucose, small amount of cystine and phenylalanine and adequate aeration. The results were not, however, established by actual viable count, method as standardised by Sokhey (12). Casein hydrolysate contains the three essential amino-acids mentioned above, but in order to find out what other elements should be provided in the media a preliminary qualitative analysis of the virulent plague organism was carried out and the bacterial bodies gave positive reactions to C, H, N, O, P, S, and fat; the ash gave positive test for Na, K, Mg and Ca, the last two in traces only. It was, therefore, to be seen whether some mineral compounds e.g., Fe, P, Mg, and vitamin, special amino-acids etc. would stimulate the growth of *P. pestis* when added to the casein hydrolysate basal medium and thus improve both quality and quantity of vaccine and the yield of the specific protective substance. At least, the growth (according to 48 hours viable count) should be equal to, if not exceeding that of the nutrient broth.

*Minerals and other requirements of the media :*

According to the analysis of bacterial bodies of *P. pestis* the elements C, H, N, O, P and S could be supplied by the amino-acids contained in the casein-hydrolysate. Na was available from NaCl as well as  $\text{Na}_2\text{HPO}_4$  contents and K from  $\text{KH}_2\text{PO}_4$  added to the medium. It is required to be seen whether the traces of calcium needed could be met from the  $\text{CaCl}_2$  added for deferretion while the magnesium requirement was left to be determined experimentally.

*Iron and phosphates :*

Iron does not actually enter into the constitution of plague bacillus, but it gets into media during hydrolysis from various sources like commercial acids, crude  $\text{BaCO}_3$  or even casein. It is removed by the method of deferretion. But it is known that iron salts can bring about a reduction of molecular oxygen in the culture medium similar to that of sodium sulphite,  $\text{CaSO}_3$ , blood etc. or even act as a catalase. Also, Rao in his studies on the metabolism of plague bacillus found haematin as an active compound that reduced the lag in the growth of plague bacillus. Haematin is an iron prophyrin compound with pyrrole structure and

this probably explains the indispensibility of the amino-acid proline which has also a pyrrole nucleus and the reason why blood provides the best medium for the growth of the organism (Gore, 16, 17; Webster, 18; and Sokhey, 12). Thus it is a question whether the effect of haematin in stimulating the growth is due to the ability of its iron content to keep down the oxygen tension or it is due to its effect as a coenzyme postulated by Rao (9). Perhaps in case of haematin both the functions are effective. It was, therefore, of importance to see the effect of iron as a growth stimulant. The effects of removal and non-removal of iron and of absence of phosphates are shown in Table VI

TABLE VI  
48 hours viable counts of the media with or without removal of iron and with or without addition of phosphates.

Lot No.	Basal crude hydrolysate without iron removed and without addition of $\text{PO}_4$ normal $\text{PO}_4$ content = 0.037%	Basal crude hydrolysate with Fe not removed and $\text{PO}_4$ added ( $\text{PO}_4$ content = 0.097 + 0.037% = 0.134%)	Basal hydrolysate with iron removed and $\text{PO}_4$ added ( $\text{PO}_4$ content = 0.076 + 0.03, = 0.113%)
I	301	249	320
XI	276	250	320
Average	288	250	320

The actual phosphate content of the casein hydrolysate basal medium with 2.7 mg N/ml was between 0.129 to 0.134 mg. per ml. From the results given in table VI, the amount seemed to be high for plague growth, particularly if iron was not removed. It was, therefore, thought that a little more than just the quantity of phosphates (both Na and K) required for the removal of iron would be sufficient for the media. Thus addition of 0.2 mg. of  $\text{Na}_2\text{HPO}_4$  and 0.067 mg of  $\text{K}_2\text{HPO}_4$  per mg of N in the medium would after deferretion with 0.04 mg of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , leave behind 0.027 + 0.037 or 0.064 mg of phosphates in the final medium.

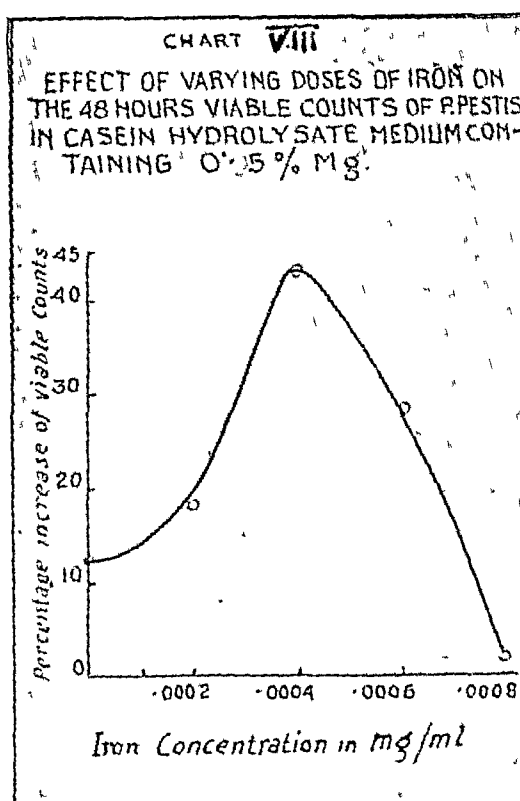
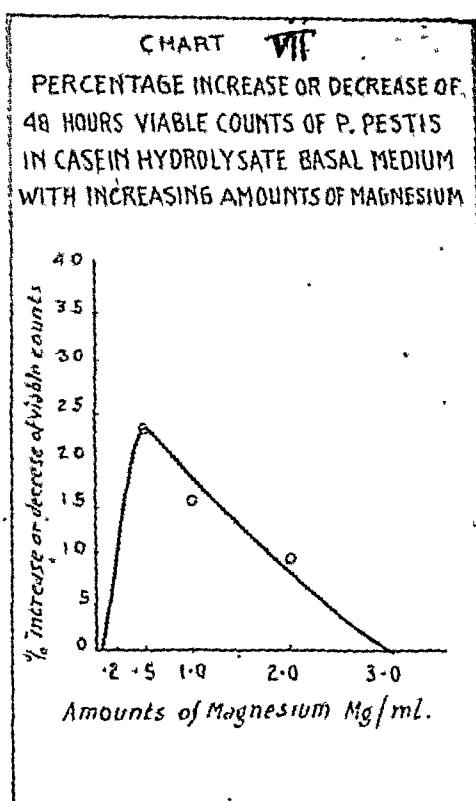
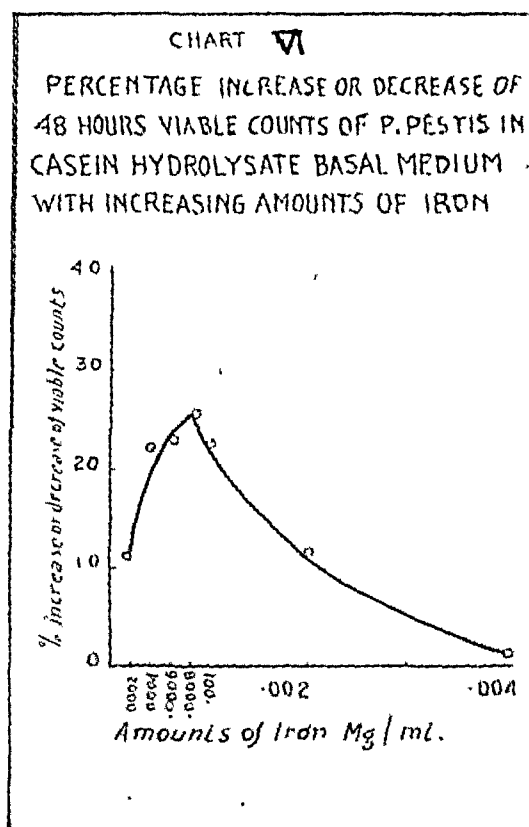
#### Iron, magnesium and calcium:

The effects of different doses of iron ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ) and magnesium ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) and of iron and magnesium combined on the 48 hours viable count are shown in Charts VI, VII and VIII. Quantities of iron between 0.0004 to 0.0008 mg. per ml of casein hydrolysate broth containing 2.7 mg N/ml gave an average increase of 26.6 per cent in the viable counts while the requirement of magnesium appeared to be in the neighbourhood of 0.5 mg per ml at which level 23 per cent increase in the viable count was recorded. An increase of 42.4 per cent in the viable count was noted when 0.0004 mg/ml of iron was added in the presence of 0.05% magnesium.

Calcium was found only as a trace in the bacterial ash of *P. pestis*. An addi-



tional amount (varying between 0.005 mg. to 0.1 mg) of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  other than what is used for defecation when tried in the presence or absence of iron did not



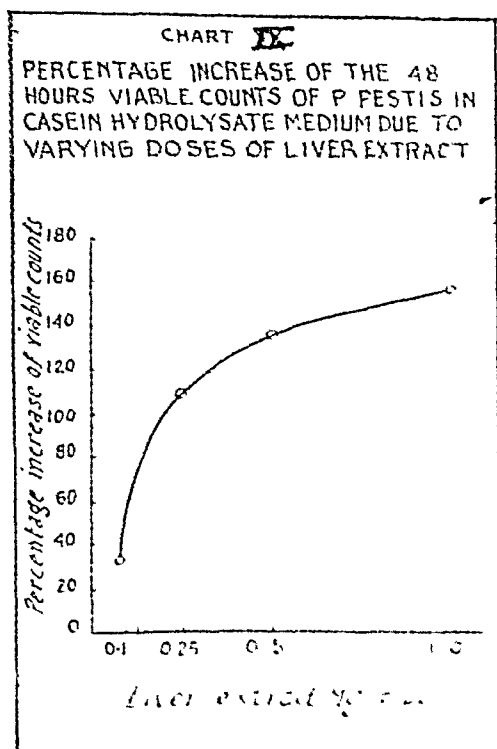
give encouraging result. On the contrary, the improvement effected by iron and magnesium was adversely affected by its addition.

*Effects of certain important amino-acids, tryptophane and cystine :*

Tryptophane is destroyed during hydrolysis of casein while Rao(9) added an extra amount of cystine (0.1 mg/ml of l-cystine) to the gelatine (hydrolysate medium to obtain better growth. Although casein normally contains more cystine (0.3%) than gelatin (0.17%), both the amino-acids were added either separately or in combination to test their effect on the viable count. It has seem that with 0.1 mg/my of cystine, the optimum for gelatin hydrolysate the improvement of growth was negligble (7.5%) and no better effect was noted with even 10 times that dose. The same result was obtained with tryptophane except with a dose as high as 2 mg/ml giving an increase of 35.8 percent in the viable count but this could be obtained with iron (0.0004 mg/ml) alone, in the presence of which both cystine and tryptophane rather showed an adverse effect. Thus, neither of them was of help in the improvement of the basal medium.

*Effect of Liver Extract :*

Rao (14) indicated nicotinic acid as one of the growth stimulating agents for *P. pestis* in a chemically defined medium. Pimelic acid and p-alanine or pantothenic acid have been found useful for the growth ad toxin production of some important bacteria. These substances are cheaply available in liver extract. It was therefore, thought worthwhile to test its effect on the growth of *P.pestis* when added in reasonably small amount to the casein hydrolysate basal medium. The results are given in Chart IX.



The sample of liver extract (of an American firm) used in the above experiment in doses of 1 mg/ml with 0.5 percent NaCl and 0.125 percent phosphates yielded 168 million per ml viable count in 48 hours at 28°C. Thus, liver extract contains substances which by themselves could support the growth of plague organism

in presence of NaCl and phosphates. When added to casein hydrolysate base there was a progressive rise of the viable counts with increasing amount of liver extract from 0.1 mg to 1 mg/ml. As high as 157.8 percent increase was obtained with 0.1 percent liver extract. The alcoholic extract of liver prepared in the laboratory gave the same increase of growth when used in 0.2 percent amount. The effect of liver extract in presence of iron and magnesium salt was then tested. The results are given in table VII.

TABLE VII

*Effect of varying doses of liver extract on the 48 hours viable count of P. pestis in casein hydrolysate medium (pH 7.4) containing different doses of iron and magnesium salts.*

No.	Basal medium	FeSO <sub>4</sub> , 7H <sub>2</sub> O	MgSO <sub>4</sub> , 7H <sub>2</sub> O	Liver extract.	Average 48 hours viable count million/ml.	Percentage increase
1	Lot IV	—	—	—	320	—
2	„	.0004 mg/ml.	—	—	435	35.9
3	„	—	0.5 mg/ml.	—	369	23.0
4	„	.0004 mg/ml.	„	—	470	42.4
5	„	—	—	0.5 mg/ml	432	35.0
6	„	.0001 mg/ml.	—	0.075 „	375	17.2
7	„	„	—	0.1 „	420	30.2
8	„	.0002 ml.	—	0.025 „	385	20.3
9	„	„	—	0.05 „	440	47.5
10	„	„	—	0.075 „	452	40.2
11	„	„	—	0.1 „	508	58.75
12	„	.0004 mg/ml.	—	0.05 „	555	73.4
13	„	„	—	0.1 „	655	104.7
14	„	.0002 mg/ml.	0.25 mg/ml.	0.05 „	467	46.0
15	„	„	0.5 „	„ „	600	87.5
16	Lot V	—	—	—	250	—
17	„	.0002 mg/ml.	0.25 mg/ml.	0.1 mg/ml. or 0.2 „ (locally prepared).	430	72.0
18	„	„	0.5 mg/ml.	„	490	96.0
19	Lot XVI	—	—	—	240	—
20	„	.0004 mg/ml.	0.5 mg/ml.	0.1 mg/ml.	530	120.4

It will be seen from Table VII that the highest growth (120 percent) was obtained when 0.1 mg of liver extract was used in combination with 0.0002 mg of iron (Fe SO<sub>4</sub>, 7H<sub>2</sub>O). and 0.5 mg of magnesium (MgSO<sub>4</sub>, 7H<sub>2</sub>O) per ml of the broth, while an increase of 96 percent was obtained by using 0.2 mg of laboratory, made alcoholic extract of liver in place of 0.1 mg of the purified American product. By reducing the liver extract to half (i.e. 0.05 mg/ml as in Expt. No. 15), for its more economical use, an increase of 87.5 percent in the growth was obtained. On the latter basis the total amount of liver extract needed for 10,000 litres would be 500 grammes or, if 0.1 mg/ml is used, 1000 grammes (roughly 2 lbs.).

#### COMPOSITION OF THE FINAL MEDIUM

In accordance with the results obtained in the above experiments the final medium was prepared as follows:

Milk casein was hydrolysed by the combined HCl and H<sub>2</sub>SO<sub>4</sub> acid method of Mueller and Johnson (6) for 72 hours, neutralised, filtered and nitrogen content

estimated by microKjeldahl technique. To the filtrate 0.2 mg of anhydrous  $\text{Na}_2\text{HPO}_4$  and 0.067 mg of  $\text{KH}_2\text{PO}_4$  were added per mg of N, followed by 0.04 mg of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . The pH was adjusted between 7.4 and 7.6 with NaOH and the mixture heated for 5 minutes. The precipitate thus formed was filtered off through paper. The filtrate was then diluted to contain 2.7 mg N/ml and then  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and liver extract were added in doses of 0.5 mg, 0.0004 mg and 0.1 mg respectively per ml of the medium and the pH readjusted, if necessary. The medium was then distributed in flasks and sterilised in the autoclave at  $110^\circ\text{C}$  for 10 minutes.

#### RATE OF GROWTH OF *P. pestis* AND THE LAG PERIOD

Perhaps the best criterion of a good medium for any organism is abundant growth and the minimum or absence of lag period. To test the efficiency of casein hydrolysate broth in these respects samples of *basal* media with and without liver extract were inoculated with a known number of organism from an actively growing 6 hours seed culture\* and incubated at  $28^\circ\text{C}$ . Viable counts were made after varying intervals of incubation beginning from  $\frac{1}{2}$  hour to 54 hours. A sample protocol of the results obtained is given in table VIII.

TABLE VIII

*The progress of growth of P. pestis in casein hydrolysate media in relation to the periods of incubation.*

Periods of incubation.	Viable counts per ml.	
	Casein hydrolysate basal.	Casein hydrolysate with liver extract.
(seed)	536	420
$\frac{1}{2}$ hour	—	595
1 "	585	645
$1\frac{1}{2}$ "	—	915
2 hours	615	1,005
3 "	750	—
6 "	2,800	4,450
9 "	4,400	—
12 "	8,000	—
15 "	13,500	—
18 "	28,500	226,000
21 "	65,000	—
24 "	155,000	2,860,000
27 "	550,000	—
30 "	850,000	25,000,000
33 "	3,500,000	—
36 "	7,700,000	65,000,000
39 "	16,700,000	67,000,000
42 "	37,200,000	106,000,000
45 "	63,000,000	140,000,000
48 "	48,000,000	—
51 "	61,000,000	157,000,000
54 "	107,500,000	193,000,000

\* After two consecutive subcultures in nutrient broth at intervals of 48 hours the third subculture in broth was incubated for 6 hours at  $28^\circ\text{C}$  and was used as a seed. The sowing was done with 0.1 ml. of  $2 \times 10^{-3}$  dilution of this broth to 0.9 ml. of the experimental broth.

The results given in table VIII show that casein hydrolysate basal medium yielded quite a fair amount of growth practically without any lag period. Only the count at 48 hours showed a little drop but the rate of growth was quite regular and its amount comparatively larger in the hydrolysate containing liver extract. The count did not, however, come up to the normal expectation, the tubes being disturbed many times for the purposes of viable count. Since the latter was done at shorter intervals in the earlier hours of the incubation, it is clearly seen that the lag period is completely absent. Thus, the medium could be considered as fully nutritive. The results obtained here further indicate that in a fully nutritive medium the so-called lag period is absent and thus raise an important question whether the lag period in bacterial growth is a true condition or not.

SPECIFIC SOLUBLE PROTEIN OF *P. pestis* IN THE CASEIN HYDROLYSATE MEDIUM  
(PRELIMINARY TEST)

Since the chief object of a protein-free medium was the isolation of a specific soluble protein in a pure form from the culture of plague bacillus, it was necessary to find out the efficiency of the casein hydrolysate in this respect.

Four flasks of the hydrolysate containing the required amount of iron and magnesium and the minimum amount of liver extract (0.1 mg/ml) were inoculated with the seed culture of *P. pestis* and incubated at 28°C. The flasks were removed one at a time at successive intervals of one week. As the flasks were removed the contents were centrifuged and the supernatant clarified and made bacteria-free by passing through L<sub>2</sub> candles. The filtrates were then separately saturated with Na<sub>2</sub>SO<sub>4</sub> at 37°C and the precipitate collected upon a filter paper, dissolved in water and reprecipitated with Na<sub>2</sub>SO<sub>4</sub> at full saturation. It was then taken up in water and dialysed till sulphate-free and finally dried in a desiccator over P<sub>2</sub>O<sub>5</sub>. The highest yield was from the sample removed at the end of 3 weeks. Each of them was then dissolved in 0.85 percent NaCl solution at pH 7.2-7.4 so as to give 1 mg of the substance per ml. The actual dilution was determined by nitrogen estimation. Higher dilutions of the protein were made and 0.1 ml of each of these dilutions was mixed with 0.5 ml of 1:10 dilution of a potent antiplague horse serum (H 23), incubated at 37°C for 2 hours and then left overnight in the refrigerator. The results, read next morning, are given in table IX.

TABLE XI

*Results of precipitation reaction of the specific soluble protein of P. pestis against H23, antiplague serum (1:10)*

Protein from culture incubated for	Dilutions of specific protein (crude)					
	4000	8000	16000	32000	64000	128000
1 week	++	++	+±	+±	+	+(f)
2 weeks	+++	++±	++	+	+	+(f)
3 weeks	+++	++±	++	+±	+	+
4 weeks	+++	+++	+±±	++	+±	+

It will be seen from table IX that the crude proteins isolated from the casein hydrolysate medium gave a specific precipitation reaction in fairly high dilution

of 1 : 128000 and that there was very little qualitative difference amongst them due to the period of incubation. Thus this preliminary test gave highly encouraging results for the isolation of soluble specific protein from the plague bacillus.

#### COMPARATIVE MOUSE-PROTECTIVE AND TOXIC DOSES OF THE VACCINES PREPARED IN THE CASEIN HYDROLYSATE AND OTHER MEDIA

The mouse-protective doses of the usual Haffkine broth vaccine and of vaccines prepared from the 3 days' as well as 4 weeks' growth in casein hydrolysate broth at 28°C and from the 3 days' growth on agar at 37°C were estimated by Sokhey (19, 20) at the Haffkine Institute, Bombay. The results are given in table X.

TABLE X

*Mouse protective doses of the vaccines prepared and maintained in different ways.*

Types of vaccine	Mouse protective doses.
1. Haffkine broth vaccine, 4 weeks' growth at 28°C, heat-killed (54°C for 15 minutes)	0.0065 ml.
2. Casein hydrolysate broth vaccine, 4 weeks' growth at 28°C, 0.1% formalin killed	0.004 ml.
3. „ stored at 0°C for 8 months	0.004 ml.
4. „ stored at 37°C for 8 months	0.004 ml.
5. Agar vaccine, 3 days' growth at 37°C, heat-killed at 54°C for 15 minutes (1000 million organisms per ml.)	0.0048 ml.
6. „ stored to 0°C for 8 months	0.004 ml.
7. „ stored at 37°C for 8 months	0.006 ml.
8. Casein hydrolysate broth—3 days' growth at 28°C (1000 million per ml.)	.0053 ml.

From the results given table X, it is apparent that the vaccine prepared in the casein hydrolysate broth is more potent than the Haffkine broth vaccine and that the potency remains unaffected by storage at 37°C for 8 months. Even three days' growth in this medium gives a smaller mouse-protective dose than the Haffkine broth vaccine and nearly as good as the agar vaccine.

#### TOXICITY OF DIFFERENT TYPES OF PLAGUE VACCINE

One of the objectionable features of the Haffkine plague vaccine has been its toxicity. As a matter of fact the original conception of potent vaccine was to depend upon its toxic action. The toxic dose of casein hydrolysate broth vaccine has been compared with that of agar-grown and Haffkine broth vaccines and the results are given in table XI. The toxic dose was taken to be the one which killed half the number of mice used.

TABLE XI

*The toxicity and mouse protective doses of three different plague vaccines.*

Types of vaccine	Mouse protective dose	Toxic dose.
Haffkine broth vaccine	.006 ml.	0.2 ml.
Casein hydrolysate broth (1000 million organism per mill 3 days' growth)	.0053 ml	0.8 ml.
Agar vaccine (1000 million organism per ml.)	.0048 ml.	1.8 ml

The toxicity of the casein hydrolysate broth vaccine is of low order. The medium also gives negative biuret reaction and an intradermal injection does not produce any kind of reaction.

### COST OF PRODUCTION

The costs of production of the acid-digested mutto broth as was being used in the Haffkine Institute, Bombay and of the casein hydrolysate according to the market value of the ingredients (in 1942-43) were calculated for 10,000 litres, the then approximate annual requirement of the Institute. It was seen that even with the most expensive chemicals of C. P. American quality the cost of production of casein hydrolysate was almost half of what was being spent for the production of acid-digested mutton broth. Much labour and time could also be saved by using P-fundler glass-lined steam heater with condenser, of 100 or multiples of 100 litre capacity. Several of them are now being used in the Haffkine Institute but it involved a certain amount of capital lay out.

### DISCUSSION

The main object of the above investigation was to evolve a protein-free medium which would (1) give an abundant growth of virulent *P. pestis* with its full antigenic constituents, (2) facilitate the isolation of specified protein substance in a more or less pure form, and (3) effect an economy in the cost of production of antiplague vaccine.

Although milk casein hydrolysed by the combined  $\text{H}_2\text{SO}_4$  and  $\text{HCl}$  method of Mueller and Johnson (6) could not be made completely protein-free even after 192 hours digestion the 72 hours digest yielded only 0.8 per cent of the total nitrogen precipitable as protein at 50 per cent saturation with  $\text{Na}_2\text{SO}_4$  and the maximum recovery of nitrogen e.g. 97.6 per cent, of which about 63 per cent was in the form of amino-nitrogen. Parallel experiments carried out with the 2.6 N  $\text{H}_2\text{SO}_4$  digest of White and Elman (8) proved in many respects inferior to the combined acid treatment except in the matter of retaining some amount of tryptophane which, fortunately, is not an essential amino-acid for the growth of virulent *P. pestis*. Crude  $\text{BaCO}_3$  was found as efficient as  $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$  for neutralising  $\text{H}_2\text{SO}_4$  after digestion.

The cost of production was also found to be only half of what was being spent for the acid-digested mutton broth at the Haffkine Institute, Bombay.

The average 48 hours viable counts in the four media viz., acid-digested mutton broth, protein-free gelatin hydrolysate, protein-free casein hydrolysate basal medium and nutrient broth were 150, 160, 320 and 400 million per ml respectively. Thus compared to nutrient broth 72 hours casein hydrolysate basal medium nutritionally needed further improvement.

A preliminary quantitative analysis revealed that the virulent *P. pestis* was composed of the following elements, viz., C, H, N, O, S, P, Na, K and traces of Mg and Ca. Although it is possible for the bacteria to synthesize certain complex substances needed for the metabolism from the simple substances provided in the medium, the presence of all the elements, stated above, would perhaps be helpful. The casein hydrolysate, however, provided the three essential amino-acids viz.,

proline, phenylalanine and cystine, as studied by Rao (9), besides other amino-acids except tryptophane, thus supplying the elements C, H, O, N, S and P. The optimum nitrogen concentration was found to be around 2.7 mg/ml. which also allowed an automatic adjustment of its NaCl content to 0.5 to 0.6 per cent.

Of the mineral requirements Na and K were provided in the form of NaCl, Na and K—phosphates and calcium was mainly added for deferrection. Iron, though not directly necessary, was probably helpful in controlling the oxygen tension in the same manner as sodium sulphite, blood, haematin etc. or may have some catalytic effect. It seems, however, that if in the crude medium the iron content was kept within the range of 0.0004 to 0.0008 mg. per ml. of the medium containing 2.7 mgN/ml. the step adopted for deferretion could be omitted and calcium could be reduced to one-tenth of the amount required for deferretion. Mg was supplied as  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  which, in combination with  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , gave a 48 hours viable count of 450 million/ml. as against 400 million/ml. given by the nutrient broth.

Alcoholic extract of liver (antianæmic factor), when added in as little an amount as 0.05 mg/ml. or 0.1 mg/ml. in the presence of iron and magnesium, increased the viable counts by 90-120 per cent and the lag period was practically eliminated, the evidence of growth being apparent even within  $\frac{1}{2}$  hour. It is not yet known which of the constituent or constituents in the liver extract actually stimulated the growth except the suggestion of nicotinic acid by Rao (14) and its probable iron sparing action as observed in the present studies. Further work in this line is, therefore, indicated.

The yield of specific protein substance was also very encouraging; the crude protein isolated from the supernatant of the growth of *P. pestis* in this medium for various periods gave positive precipitin reaction against a potent antiplague serum at a high dilution of 128,000.

On the whole, the nutritive requirement of *P. pestis* seem to be simple amino-acids and some inorganic salts. Berkman (21) in his recent studies on the accessory growth factors for the growth of *pasteurella* group of organism came to the same conclusion although only laboratory strains were used and the technique was not standardised. Antiplague vaccines prepared in the casein hydrolysate broth in the Haffkine Institute have been found less toxic and more potent than hitherto prepared broth vaccine. It also possesses high keeping qualities and is thus eminently suited for the preparation of plague vaccine.

#### SUMMARY

1. A protein-free liquid medium prepared from commercial milk casein by  $\text{H}_2\text{SO}_4 + \text{HCl}$  combined acid hydrolysis for 72 hours, neutralised by crude  $\text{BaCO}_3$  and NaOH, and containing 0.5-0.6 per cent NaCl and 2.7 mg. of N/ml., yielded a fairly good growth of *P. pestis*, but as a basal medium the growth did not come up to the standard equal to that given by the nutrient broth.

2. Supplementation with a little mangesium and iron was necessary to bring the growth up to a standard which could compare favourably with that of nutrient broth. A further improvement in the amount of growth was obtained by the addition of a little quantity of liver extract. In this connection the doses of iron and phosphates have been discussed.



3. The final medium was prepared as follows:

To casein hydrolysate crude base 0.2 mg. of  $\text{Na}_2\text{HPO}_4$  and 0.06 mg. of  $\text{KH}_2\text{PO}_4$  were added followed by 0.04 mg. of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  per mg. of N/ml. The pH was adjusted to 7.4-7.6 by means of NaOH and the mixture heated for 5 minutes. The precipitate, thus formed, was removed by paper filtration and the concentrated hydrolysate was diluted to contain 2.7 mg. N/ml., and then  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and liver extract were added in doses of 0.5 mg., 0.0004 mg. and 0.1 mg. respectively per ml. of the medium. The pH was readjusted if necessary and the medium distributed in flasks and sterilised in the autoclave at  $110^\circ\text{C}$  for 10 minutes.

4. Such a medium not only eliminated the so-called lag period and effected an increase in the viable count, being evident within half an hour from the time of inoculation, but also yielded an active specific soluble protein substance in the culture filtrate of the plague organism and thus opened up a fresh approach to the study of the subject.

5. The plague vaccine prepared in the medium was found superior to Haffkine broth vaccine and was less toxic and possessed high keeping qualities.

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CASEIN HYDROLYSATE AGAR—A NEW SOLID MEDIUM  
FOR THE GROWTH OF *PASTEURELLA PESTIS*  
AND ALLIED ORGANISMS.\*

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The difficulties encountered in the enumeration of viable plague organism by the method of plating led Sokhey (1) to reinvestigate the problem of finding a suitable solid medium for the growth of *P. pestis*. Of the various media tried he found 5 per cent rabbit-blood agar as the best medium for the purpose, the optimal temperature for incubation being 37.5°C. Gore (2, 3) only tentatively came to the same conclusion, as his findings were not supported by actual viable count under standardised conditions.

Casein hydrolysate broth as standardised by Seal and Mukherji (4, 5) proved fully nutritive and so also was the nutrient broth. But the latter did not prove so efficient when it was made into a solid medium by the addition of agar. On the other hand, a solid medium to replace blood agar would eliminate the trouble of procuring rabbit's blood on a large scale. Besides, the vaccine prepared from the organisms grown on rabbit-blood agar could not be used with impunity either for human immunisation or for the production of antiplague serum in horses. Casein hydrolysate agar was, therefore, prepared to test its efficiency for the growth of *P. pestis* so that, if found suitable, it could be used (1) as a solid medium to replace blood agar for (a) colony count, (b) preservation of virulent *P. pestis*, (c) preparation of bacterial suspension for quantitative agglutination, virulence and animal protection tests and (d) preparation of antiplague vaccine. It would also (2) facilitate the study of the chemical antigenic structure of the plague organism namely, specific proteins (from the autolysates) and polysaccharides. The results obtained are presented below:

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\*This was carried out in 1941 at the Haffkine Institute, Bombay and was presented before the 37th Session of the Indian Science Congress Association, held at Poona in January, 1950.

## EXPERIMENTAL

*Preparation of the medium*

To a litre of casein hydrolysate basal medium, neutralised, clarified and diluted to contain 2.7 mg. of nitrogen per ml., 0.2 g. of anhydrous  $\text{Na}_2\text{HPO}_4$  and 0.06 g. of anhydrous  $\text{KH}_2\text{PO}_4$  were added followed by 0.04 g. of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (in the form of 10%  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) and the pH adjusted to 7.4–7.6 with concentrated NaOH; the mixture was heated for 5 minutes and the precipitates, thus formed, removed by filtration through paper; then 0.5 g. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.4 mg. of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.4 ml. of 0.1 per cent solution) were added followed by 0.1 g. of purified liver extract or 0.2 g. of crude alcoholic extract of liver (prepared in the Laboratory).

To the above broth 25 g. of agar were added and dissolved by steaming. The pH was adjusted to 7.4–7.6 and the mixture was distributed in Roux bottles, Legroux tubes or test tubes, as necessary, sterilised in an autoclave at  $110^\circ\text{C}$  for not more than 20 minutes and slanted for solidification. The prepared media were then placed in the hot room ( $37.5^\circ\text{C}$ ) overnight to test their sterility. Final sterilisation needed careful supervision as over-heating at a higher temperature or for a longer period resulted in the deterioration of its efficiency. The layer of the medium of Roux bottles should neither be too thick nor too thin. An amount between 150 to 200 ml. was found suitable to meet the requirement.

*Experimental Procedures*

Two strains, 139/L and 337/L of *P. pestis*, isolated from human cases of bubonic plague in 1940 at Latur, Hyderabad State, and maintained in the refrigerator as the first subculture of rabbit-blood agar, in a sealed condition, were used. The organisms were highly virulent requiring only 6 to 7 organisms to kill 80 per cent of the Haffkine Institute-bred mice in 7 days.

*Inoculation of Legroux Tubes For Viable Counts*

The same procedure as adopted by Sokhey (1, 6) was employed. Seven progressive tenth dilutions were made from a standard 48 hours broth culture of *P. pestis*, 139/L at  $28^\circ\text{C}$ , and Legroux tubes, 4 in each series, containing rabbit-blood agar, casein hydrolysate agar or nutrient agar were each seeded with 0.05 ml. of  $10^{-5}$  and  $\frac{1}{2} \times 10^{-6}$  dilutions of the culture in separate series. They were then incubated at  $37.5^\circ\text{C}$  for 72 hours.

*Inoculation of Roux Bottles*

The Roux bottles containing casein hydrolysate agar or nutrient agar were inoculated with 12–15 ml. of 48 hours seed culture made in casein hydrolysate broth either in separate test tubes or in the Haffkine balloon flask of 250, 500 or 1 litre capacity. The bacterial suspension was well spread over the surface of the media and the bottles incubated at  $37.5^\circ\text{C}$  for 48 hours with agar surface on the top so that the excess of broth left over after spreading the inoculum on the agar surface could supply the moisture needed for giving a heavy growth. The casein hydrolysate agar in Legroux and test tubes was used in the same way as blood agar by Sokhey (1, 6).

## RESULTS

*Growth In Roux Bottles*

The growth in 25 Roux bottles inoculated and incubated in the above manner was washed with physiological salt solution. Altogether 10 litre of emulsion of 1,000 million/ml. was obtained i.e. equivalent to 10,000 doses of agar-grown vaccine of 1000 million organisms per ml.

The total amount of casein hydrolysate broth used for 25 flasks was 3750 ml. in which 93.75 g. of agar was added. When broth alone was inoculated and incubated for 72 hours, it yielded 1000 million organisms per ml (Sokhey, 7) i.e. 3750 doses of vaccine of the above strength. In other words, the total yield was more than  $2\frac{1}{2}$  times more in the casein hydrolysate agar than in the broth.

This yield was found much more favourable when it was compared with the yield of vaccine in nutrient agar. From 600 ml. of casein hydrolysate broth 600,000 million (i.e. equal surface area) yielded 160,000 million organisms or only 160 doses of 1000 million/ml. (Sokhey, 7). In other words, the total yield expected from 3750 ml of nutrient agar was only 1000 doses of vaccine of the above strength as against 10,000 doses in casein hydrolysate agar. Accordingly, the latter was 10 times more efficient than the nutrient agar.

As the results were found encouraging this medium was later used in place of rabbit-blood agar for growing plague organisms to carry out immunization, absorption, cross-absorption and agglutination tests and to study their specific proteins and polysaccharides from the autolysates with good results, to be reported in subsequent communications.

*Viable count of P. pestis in casein hydrolysate agar*

Since 5 per cent rabbit-blood agar gave consistent viable counts at a dilution of  $10^{-6}$  of the 48 hours growth of *P. pestis* in nutrient broth at  $28^{\circ}\text{C}$  three dilutions, namely,  $10^{-3}$ ,  $10^{-6}$  and  $0.5 \times 10^{-6}$  were used for comparing the efficiency of casein hydrolysate agar with that of rabbit-blood agar. The results are given in table I.

TABLE I

*Viable counts\* of P. pestis on casein hydrolysate agar compared with those on rabbit-blood agar.*

Medium	Average number of colonies per tube in the dilutions used.		
	$10^{-3}$	$10^{-6}$	$0.5 \times 10^{-6}$
Nutrient agar	Nil	Nil	Nil
Casein hydrolysate agar	128	14	8
5% Rabbit-blood agar	144	16	9

\*Average of at least 4 counts.

It will be seen from table I that the average colony count obtained in the casein hydrolysate agar is nearly as good as in the 5 per cent rabbit-blood agar and is the best so far obtained without blood or serum in the media. Nevertheless, there is still room for further improvement.

## DISCUSSION

The difficulties of growing *Pasteurella pestis* on solid medium other than blood or serum agar without losing its full antigenic properties were overcome by the introduction of casein hydrolysate broth. Special care is, however, needed in the sterilisation of the medium which should be carried out in the minimum of time and under minimum temperature and pressure. The yield of growth is about 10 times that obtained on nutrient agar and  $2\frac{1}{2}$  times that obtained in casein hydrolysate broth. The viable counts made on this medium closely approximate those given by the rabbit-blood agar. Thus the introduction of this medium has opened up an opportunity to utilise it for the various purposes stated at the outset.

## SUMMARY

The casein hydrolysate agar medium prepared by adding 2.5 per cent agar to the standardised casein hydrolysate broth containing 0.01 per cent liver extract forms a convenient and good solid medium for the growth of *P. pestis* and allied organisms. After the agar is melted by steaming and distributed in the containers, a temperature of  $110^{\circ}\text{C}$  and a pressure not exceeding 10 pounds for 20 minutes in the autoclave have been found suitable for final sterilisation.

The obvious utilities of the medium have been indicated, though there is still scope to improve it for a more generalised use.

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